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Development of diagnostic tests for the detection of *Neospora caninum* infected cattle

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Abstract

The protozoan parasite *Neospora caninum* is among the most frequently diagnosed infectious causes of bovine abortion thus causing significant economic losses, production inefficiency and welfare concern to the cattle sector worldwide. The control of bovine neosporosis relies on management techniques within which the identification of infected animals by serological testing plays a key role. However, the reliable diagnosis of the disease is hindered by the complex host-parasite interactions and the intrinsic limitations of the serological diagnostic tools currently available; as a result, some infected animals may not be detected. At the herd, regional and national levels, this can potentially undermine efforts for the control of the disease.

The work presented in this thesis was undertaken to further investigate avenues to improve the diagnosis of *N. caninum* infection in cattle.

It has been shown previously that recombinant antigens expressed by the rapidly proliferating tachyzoite stage and the slowly multiplying bradyzoite stage of *N. caninum* can be successfully employed for the detection of specific antibody responses during acute and persistent infections respectively. Following the establishment of persistent infections, sustained by the bradyzoite stage, the antibody levels against the tachyzoite stage may decline below the detection limits of currently available diagnostic tests which are exclusively based on antigens expressed by the tachyzoite. Consequently, the use of bradyzoite antigens for the development of serological diagnostic tests, may enhance the identification of infected animals.

Novel antigens putatively expressed by the quiescent bradyzoite stage of *N. caninum* have been identified, expressed as recombinant proteins and assayed for the detection of specific antibodies. The recognition of recombinant tNcSRS12A-B and tNcSRS44-A by specific antibodies in sera from persistently *N. caninum*-infected cattle suggested that these proteins could be used for the detection of persistently infected animals.

Indirect ELISAs based on previously characterised *N. caninum* antigens, such as the tachyzoite surface protein rNcSRS2, the immunodominant dense granule protein rNcGRA7 and the bradyzoite specific surface antigens rNcSAG4, rNcBSR4 and rNcSRS9, as well as a commercial test using whole tachyzoite lysate as antigenic preparation, were evaluated within a cross-sectional study to estimate the

seroprevalence of bovine neosporosis in British dairy cattle. Moderate, but not high, agreement was found amongst the tests based on whole tachyzoite lysate, rNcSRS2 and rNcGRA7, and amongst the bradyzoite-specific antigen-based iELISAs. In contrast, only slight agreement was observed when each test detecting antibody responses indicative of acute infection (whole tachyzoite lysate, rNcSRS2 and rNcGRA7) was compared with each test detecting antibody responses indicative of persistent infection (bradyzoite-specific antigen-based iELISAs). Most *N. caninum* antibody-positive cattle samples showed detectable antibodies only against either antigens predominantly expressed by the tachyzoite or bradyzoite antigens thus suggesting that the exclusive use of one type of test may result in the misclassification of a proportion of animals, which test negative despite harbouring the parasite. This may result in the underestimation of the seroprevalence. Consequently, the combination of multiple tests in parallel, both tachyzoite and bradyzoite antigen-based, would improve the diagnosis of bovine neosporosis.

Molecular tools for the genetic discrimination of different *N. caninum* isolates were also investigated. A novel multilocus fragment typing (MLFT) tool based on twelve highly polymorphic microsatellite markers was developed and applied to the analysis of DNA samples obtained from laboratory-maintained *N. caninum* isolates and tissues collected from bovine foetuses aborted due to *N. caninum*. The locus-specific nested PCRs associated with automated fragment analysis by capillary electrophoresis enabled to evaluate the markers in terms of typeability and discriminatory power. Overall, the typing tool was characterised by good typeability and discriminatory power and enabled to provide information on the genetic diversity amongst the laboratory-maintained and clinical *N. caninum* isolates studied. The MLFT tool may help to investigate the likely source of infection within abortion outbreaks and aid the study of the association between the genetic heterogeneity of *N. caninum* and the diverse biological features *in vitro* and *in vivo*. Furthermore, the loci characterised by the highest discriminatory power and typeability may be used alongside already established microsatellite markers for the development of an improved typing tool which could be proposed at the inter-laboratory level.

Finally, current perceptions and common veterinary practice related to the diagnosis and control of bovine neosporosis were studied by developing a questionnaire for

cattle practitioners in the United Kingdom. The survey highlighted the awareness of the limitations of current serological techniques and the demand for additional tools in terms of diagnostics and vaccines to tackle the economic losses and animal welfare implications related to *N. caninum* in cattle.

Lay summary

Neospora caninum is a microscopic protozoan parasite which causes abortion in cattle determining significant economic losses, production inefficiency and animal welfare concern. Since there are no vaccines or treatments, the impact of bovine neosporosis (the disease caused by *N. caninum*) can only be reduced by removing infected animals from the herd or excluding them from breeding replacement stock. Indeed, the parasite can be transmitted during pregnancy from dam to foetus through the placenta.

In live animals, the infection with *N. caninum* is diagnosed by detecting specific antibodies in serum, plasma or milk. However, some animals may test negative with the antibody assays available despite being infected with the parasite.

A questionnaire-based survey for UK large animal veterinary practitioners highlighted the awareness of the limitations of current serological techniques and the demand for improved diagnostic tests and a vaccine to tackle bovine neosporosis.

The work presented in this thesis was undertaken to investigate avenues to improve the identification of *N. caninum*-infected cows. There are two life cycle stages of *N. caninum*: the slowly proliferating bradyzoite stage which establishes persistent infections and the rapidly multiplying tachyzoite stage found during acute infections. Novel antigens displayed by the bradyzoite were identified, produced using bacterial systems and used for the identification of cows which tested antibody negative with tests based on tachyzoite antigens. Most commercially available diagnostic tests use exclusively tachyzoite antigens.

Within a study to assess the prevalence of *N. caninum* antibodies in British dairy cattle, six tests using previously described tachyzoite and bradyzoite antigens were assessed and compared. Since most test-positive cows showed detectable antibodies only against either tachyzoite or bradyzoite antigens, a combination of assays using antigens of both life cycle stages of *N. caninum* should be considered to improve the diagnosis of neosporosis.

Due to the genetic variation of *N. caninum*, different strains of the parasite can be identified in a herd. A molecular technique which enables the discrimination of different strains of the parasite was developed. Characterised by high discriminatory power, this tool may be used to further investigate abortion outbreaks thus providing

an assessment of the genetic diversity of the isolates involved and additional epidemiological information.

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Author declaration

I hereby declare that this thesis is of my own work and all results presented therein have been conducted by the author between November 2013 and January 2017 under the supervision of Dr Frank Katzer, Prof. Elisabeth A. Innes (Moredun Research Institute) and Prof. Elspeth Milne (University of Edinburgh). The work has not been previously submitted for any other degree or professional qualification.

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List of abbreviations

APHA	Animal and Plant Health Agency
AI	artificial insemination
bp	base pair
<i>B. besnoiti</i>	<i>Besnoitia besnoiti</i>
<i>B. indicus</i>	<i>Bos indicus</i>
<i>B. taurus</i>	<i>Bos taurus</i>
<i>B. licheniformis</i>	<i>Bacillus licheniformis</i>
BHV	bovine herpesvirus
BSA	bovine serum albumin
BVDV	bovine viral diarrhoea virus
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CE	capillary electrophoresis
CHeCS	Cattle Health Certification Standards (CHeCS)
CNS	central nervous system
<i>C. parvum</i>	<i>Cryptosporidium parvum</i>
CPD	continuing professional development
CV	coefficient of variation
DHHPS	Dairy Herd Health and Productivity Service
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
e.g.	<i>exempli gratia</i> (for example)
ELISA	enzyme-linked immunosorbent assay
ET	embryo transfer
FBS	foetal bovine serum
HCl	hydrogen chloride
<i>H. heydorni</i>	<i>Hammondia heydorni</i>
HRP	horseradish peroxidase

IBR	infectious bovine rhinotracheitis
i.e.	<i>id est</i> (that is)
iELISA	indirect enzyme-linked immunosorbent assay
IFAT	indirect immunofluorescent antibody test
IFN- γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
IMAC	immobilised metal affinity chromatography
IPTG	β -D-1-thiogalactopyranoside
ISCOM	immune stimulating complexes
IV	intravenous(ly)
ITS1	internal transcribed spacer 1
KCl	potassium chloride
kDa	kilodalton(s)
LB	Luria Bertani
Mabs	monoclonal antibodies
MAT	modified agglutination test
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MCMC	Markov chain Monte Carlo
mRNA	messenger ribonucleic acid
μ g	microgram(s)
μ l	microlitre(s)
mg	milligram(s)
ml	millilitre(s)
mM	millimolar
min	minute(s)
M	molar
mM	millimolar
mAb	monoclonal antibody
MRI	Moredun Research Institute
NAT	<i>Neospora</i> agglutination test

NaCl	sodium chloride
NaOH	sodium hydroxide
NaHCO ₃	sodium bicarbonate
<i>N. caninum</i>	<i>Neospora caninum</i>
<i>N. hughesi</i>	<i>Neospora hughesi</i>
ng	nanogram(s)
NK	natural killer cell
OD	optical density
OIE	World Organization for Animal Health (Office International des Épizooties)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline, 0.05% Tween®20
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
RESAS	Scottish Government's Rural and Environment Science and Analytical Services Division
RNAse	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
s	second(s)
SAC	Scotland's Agricultural College
<i>S. cruzi</i>	<i>Sarcocystis cruzi</i>
SC	subcutaneous(ly)
SD	standard deviation
SDS	sodium dodecyl sulphate
Se	sensitivity

Sp	specificity
S/P	sample to positive percentage
SID	Simpson's index of diversity
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TMB	3, 3', 5, 5'-tetramethylbenzidine
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
TNF- α	tumour necrosis factor alpha
UK	United Kingdom of Great Britain
USA	United States of America
VIDA	Veterinary Investigation Diagnosis Analysis
w/v	weight/volume

Chapter 1: General introduction

1.1 *Neospora caninum*

Neospora caninum (Apicomplexa: Coccidia) is an obligate intracellular tissue-cyst forming eukaryotic parasite belonging to the phylum Apicomplexa (Dubey and Schares 2011, Dubey *et al.*, 2007). Over six thousand species of parasitic protozoans which have, as a defining feature, a characteristically polarised cell structure with an apical complex (Adl *et al.*, 2007) are included within the Apicomplexa. The apical complex is a cytoskeletal arrangement composed of a set of structural and secretory elements that are instrumental in the host cell attachment and invasion processes (Gubbels and Duraisingh 2012, Kemp *et al.*, 2013).

The phylum encompasses major veterinary pathogens such as *Eimeria* spp., an economically significant pathogen in the modern poultry industry (Quiroz-Castaneda and Dantan-Gonzalez 2015), *Toxoplasma gondii*, *Cryptosporidium* spp., *Theileria* spp., *Babesia* spp., *Beisnoitia* spp. and *Sarcocystis* spp., all of which cause clinical disease and economic losses in a variety of livestock hosts. Several apicomplexans are also important zoonotic pathogens. *T. gondii*, a primary abortifacient in sheep, is a leading opportunistic parasite associated with congenital and neurological birth defects in humans (Weiss and Dubey 2009). Certain species of the genus *Cryptosporidium* can cause neonatal enteritis in many animal species and can cause waterborne illness in people (Efstratiou *et al.*, 2017).

Initially reported in Norway in 1984, as an unidentified protozoan parasite causing severe encephalomyelitis and myositis in domestic dog puppies (Bjerkas *et al.*, 1984), *N. caninum* was formally recognised and named in 1988 after the retrospective examination of dog tissues (Dubey *et al.*, 1988a) and the *in vitro* isolation of the parasite (Dubey *et al.*, 1988b).

The recognition of *N. caninum* was initially controversial due to the close resemblance to *Hammondia heydorni*: a coccidian parasite sporadically found in dog faeces that has limited clinical significance. It was argued that the two species referred to the same parasite (Mehlhorn and Heydorn 2003). Evidence of biological, morphological and

molecular differences between the two organisms confirmed the validity of the genus *Neospora* (Dubey *et al.*, 2002b, McAllister 2000) which also includes *Neospora hughesi* (Marsh *et al.*, 1998). Despite being ultrastructurally very similar to *N. caninum*, *N. hughesi* shows distinct differences in the sequence of the internal transcribed spacer 1 (ITS1) region of the genome (Marsh *et al.*, 1998) and within the amino acid sequences of two immunodominant surface antigens (Marsh *et al.*, 1999). *N. hughesi* is associated with myeloencephalitis in horses (Howe *et al.*, 2014).

Early reports of *N. caninum* showed striking morphological and developmental analogies with *T. gondii*. This may have resulted in *N. caninum* infections being frequently misdiagnosed as *T. gondii* prior its formal recognition (Bjerkas and Dubey 1991). In subsequent years, key differences between the two parasites regarding their natural host range, pathogenesis, virulence factors and antigenicity were observed (Dubey *et al.*, 2002a, Dubey and Lindsay 1996). More recently, divergences between the two closely related apicomplexans, which are estimated to have diverged from a common ancestor around 28 million years ago, were described by extensive comparative genomic and transcriptomic analyses (Reid *et al.*, 2012).

Only a few years after clinical disease was described in dogs, cases of congenital protozoan infections causing encephalomyelitis were reported in calves (O'Toole and Jeffrey 1987, Parish *et al.*, 1987). Later, *N. caninum* was associated for the first time with an abortion outbreak in a dairy farm in the USA (Thilsted and Dubey 1989). The isolation of the parasite from aborted bovine foetuses (Conrad *et al.*, 1993a) and the experimental reproduction of foetal infection that resulted in foetal death (Barr *et al.*, 1994) confirmed the pathogenic nature of *N. caninum* in cattle.

N. caninum quickly emerged as one of the most frequently diagnosed infectious causes of bovine abortion (Obendorf *et al.*, 1995). Currently bovine neosporosis (i.e. the disease caused by *N. caninum* in cattle) is thought to have a global distribution and is considered a primary bovine reproductive problem (Goodswen *et al.*, 2013), responsible for significant economic losses and production inefficiency to the cattle sector worldwide (Larson *et al.*, 2004, Reichel *et al.*, 2013). A comprehensive review of the published data estimated the global economic impact of bovine neosporosis to exceed one billion US\$ (Reichel *et al.*, 2013).

1.2 Life cycle, host spectrum and infectious stages

1.2.1 Heteroxenous life cycle: definitive and intermediate hosts

N. caninum is characterised by a facultative heteroxenous life cycle involving a definitive host, in which the sexually mature stage of the parasite occurs, and an intermediate host in which the parasite undergoes some developmental changes and multiplies (Figure 1.1).

The definitive host range of *N. caninum* is restricted (Dubey *et al.*, 2017). Dogs (*Canis lupus familiaris*) (McAllister *et al.*, 1998) and related canids such as coyote (*Canis latrans*) (Gondim *et al.*, 2004c), dingo (*Canis lupus dingo*) (King *et al.*, 2010), and wolf (*Canis lupus*) (Dubey *et al.*, 2011) act as definitive hosts. Despite the detection of specific antibody responses, parasite DNA or both in other wild canids, such as red foxes (*Vulpes vulpes*) (Bartley *et al.*, 2013b, Stuart *et al.*, 2013), South American grey foxes (*Dusicyon griseus*) (Martino *et al.*, 2004) and two genera of canids native to South America (*Lycalopex gymnocercus* and *Cerdocyon thous*) (Canon-Franco *et al.*, 2004, Gondim *et al.*, 2004b), none of these species has been confirmed as a definitive host for *N. caninum* to date.

In red foxes, experimental infection through feeding *N. caninum*-infected tissues did not result in shedding of parasite oocysts (Schaes *et al.*, 2002b) and no experimental data were produced for the other canid species suspected to act as definitive hosts (Donahoe *et al.*, 2015, Rosypal and Lindsay 2005).

Cattle (*Bos taurus*) and a wide range of animal species, both domestic (reviewed in Dubey and Schaes 2011) and wild (reviewed in Donahoe *et al.*, 2015) can act as intermediate hosts. In contrast to the current view that *N. caninum* has a more restricted intermediate host spectrum compared to *T. gondii*, which infects almost any warm-blooded vertebrates, there is increasing evidence that the host range may potentially include most warm-blooded animals.

In addition to cattle, proven intermediate hosts, from which viable *N. caninum* was successfully isolated, include: sheep (*Ovis aries*) (Dubey *et al.*, 1990), water-buffalo (*Bubalus bubalis*) (Reichel *et al.*, 2015), European wood buffalo (*Bison bonasus*) (Bien *et al.*, 2010), white-tailed deer (*Odocoileus virginianus*) (Vianna *et al.*, 2005)

and axis deer (*Axis axis*) (Basso *et al.*, 2014). In goats (*Capra hircus*) (Dubey *et al.*, 1992a), alpacas (*Vicugna pacos*), llamas (*Lama glama*) (Serrano-Martinez *et al.*, 2007a) and several other species viable parasites were not isolated, although clinical neosporosis was confirmed. The detection of *N. caninum* DNA has been reported in tissues of a multitude of animals including rodent and avian species (Dubey *et al.*, 2017).

Dogs and cattle maintain the domestic life cycle of *N. caninum* which is of primary clinical and economic interest and has been extensively investigated. However, a sylvatic life cycle maintained by wild canids and wild ruminants is also possible (Almeria 2013, Rosypal and Lindsay 2005). In areas in which domestic and wild hosts coexist, it is likely that the two cycles overlap; nevertheless, the significance of wildlife as a reservoir of *N. caninum* has not been clarified to date (Almeria 2013).

In contrast to *T. gondii*, one the most common parasitic zoonoses worldwide (Tenter *et al.*, 2000), there is no definitive evidence that *N. caninum* can infect or cause disease in humans (Barratt *et al.*, 2010). A serological survey showed no evidence of human exposure to *N. caninum* in the general population and a cohort of farm workers in England (McCann *et al.*, 2008) suggesting that neosporosis in healthy humans is unlikely. Nevertheless, the detection of anti-*N. caninum* antibodies in human sera was reported by other studies (Ibrahim *et al.*, 2009, Lobato *et al.*, 2006, Tranas *et al.*, 1999). One of these described a significantly higher *N. caninum* seroprevalence amongst people infected with human immunodeficiency virus (HIV) or diagnosed with neurological disease compared to healthy individuals (Lobato *et al.*, 2006). Consequently, the potential role of *N. caninum* as an opportunistic organism in immunocompromised patients and a possible link to neurological illnesses has been hypothesised (Lobato *et al.*, 2006).

Oocysts measure approximately 10×12 µm in diameter and are characterised by a robust 0.6-0.8 µm-thick wall which would confer resistance to drying and freezing allowing prolonged persistence in the environment and survival of the sporozoites for extended periods of time (Dubey *et al.*, 2007, Uzeda *et al.*, 2007). Once the oocysts are ingested by the host, often via contaminated feed or water, excystation takes place and sporozoites are released in the small intestine. By analogy with *T. gondii* infection in the cat, it is assumed that sporozoites invade the intestinal epithelium where they develop into tachyzoites (Dubey *et al.*, 2004).

1.2.2.2 Tachyzoites

Tachyzoites are lunate-shaped with a central nucleus and measure approximately 2×6-7.5 µm (Dubey *et al.*, 2002a) (Figure 1.2). Growth and multiplication of tachyzoites is rapid and occurs asexually within host cells by endodyogeny: a process in which two daughter cells surrounded by their own membrane are assembled within the mother cell (Conrad *et al.*, 1993a). The tachyzoites can invade a wide range of nucleated cell types (Hemphill *et al.*, 2006) and disseminate throughout the body via the blood and lymphatic systems (Dubey and Schares 2006). Differences in the rate of invasion and growth, depending on the host cell type and strain of *N. caninum* have been reported (Regidor-Cerrillo *et al.*, 2011). After a phase of rapid proliferation, which was estimated to consist of approximately 20 divisions (occurring within a timeframe of approximately 3 weeks) (Goodswen *et al.*, 2013) the tachyzoites differentiate into the bradyzoite stage which also multiplies by endodyogeny but at a much slower pace (Dubey and Lindsay 1996).

1.2.2.3 Bradyzoites and tissue cysts

Bradyzoites are slender, variable in size (6.5×1.5 µm on average) with a terminally located nucleus (Dubey *et al.*, 2004). Unlike tachyzoites, they contain a higher number of amylopectin granules (Speer *et al.*, 1999) which stain red with the periodic acid Schiff (PAS) reaction. Amylopectin is a storage form of glucose which represents an endogenous energy source for long-term survival. Encapsulated within intracellular thick-walled (0.5-4.0 µm) cysts (Figure 1.2), that are microscopically distinguishable from *T. gondii* tissue cysts, characterised by a thinner wall, bradyzoites can persist

within an infected host for several months or years and are thought to be able to survive for the entire life of the host (Dubey and Lindsay 2006) without causing significant clinical manifestations. However, it is unclear whether the same tissue cysts can survive for the whole life of the host or whether the long-term persistence of the parasite is due to the succession of cycles of reactivation into the tachyzoite stage followed by differentiation into the bradyzoite stage and quiescence (Dubey *et al.*, 2017).

Depending on the host and cell type parasitised, tissue cysts may contain a variable number of bradyzoites and be different sizes depending on their maturity (Dubey 2003).

Unlike tachyzoites which can develop in many different tissues, *N. caninum* bradyzoite-containing tissue cysts are generally observed in the cells of the central nervous system (CNS) (i.e. brain and spinal cord) and skeletal muscles (Peters *et al.*, 2001).

Since *N. caninum* tissue cysts are rarely found in naturally infected animals, the information available on this stage of the parasite is limited to experimental animal models. Mice (McGuire *et al.*, 1997a, McGuire *et al.*, 1997b) and gerbils (Gondim *et al.*, 2001) develop relatively few tissue cysts whereas, experimentally infected fat-tailed dunnarts (*Sminthopsis crassicaudata*) were shown to produce higher numbers (King *et al.*, 2011) thus representing a promising model for the study of tissue cyst development.

The biological mechanisms which trigger the differentiation from tachyzoite to bradyzoite and the genesis of *N. caninum* tissue cysts have not been fully elucidated to date. However, complex interactions between the parasite and the host immune responses are assumed to play a major role in the stage conversion processes (Dubey and Lindsay 1996, Vonlaufen *et al.*, 2002). Furthermore, *in vitro* models of stage conversion based on the alteration of culture conditions such as pH and temperature (Weiss *et al.*, 1999) or the exposure to chemicals such as nitric oxide (NO) and the NO donor sodium nitroprusside (Vonlaufen *et al.*, 2004, Vonlaufen *et al.*, 2002), highlighted the effect of physicochemical stress in the initiation of the tachyzoite-to-bradyzoite transformation (Eastick and Elsheikha 2010).

Bradyzoites are orally infective; the *N. caninum* life cycle is completed when tissue cysts are ingested by definitive carnivore hosts and bradyzoites are released in the small intestine (Dubey *et al.*, 2004). Currently unknown gametogonic stages of *N. caninum* are presumed to precede oocysts formation in the gut of the definitive canine host. Although schizont- and gamont-like stages of *N. caninum* were observed in small intestine histological sections from a dog pup (Kul *et al.*, 2015), Dubey and colleagues (2017) have disputed the weak evidence to support that these were *N. caninum* and confirmed that the oocyst is the only sexual stage of the parasite unambiguously identified to date.

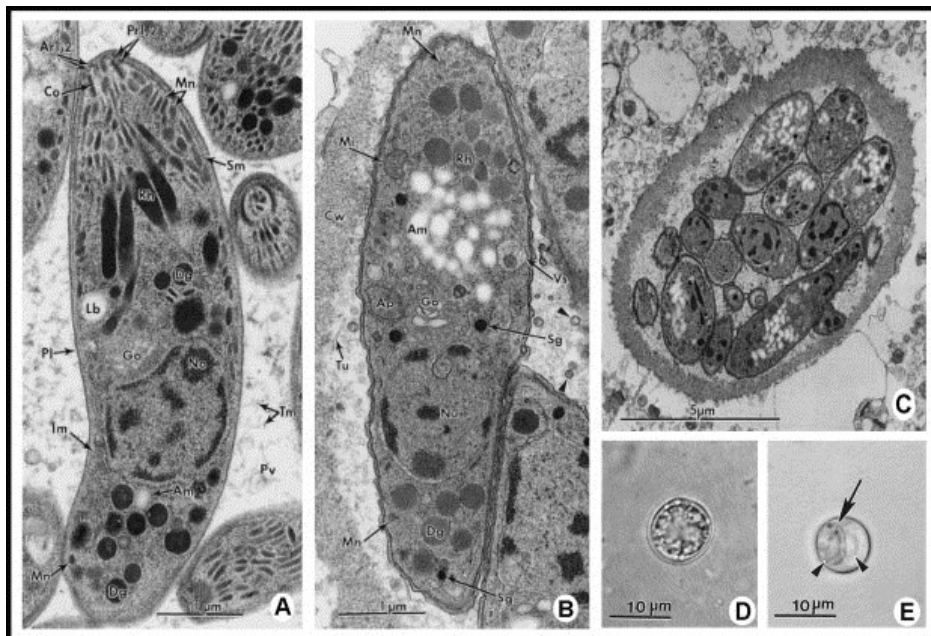


Figure 1.2 – Infectious stages of *N. caninum* (reproduced from Speer *et al.*, 1999 and McAllister *et al.*, 1998). Ultrastructure of (A) tachyzoite, (B) bradyzoite, (C) tissue cyst, (D) unsporulated oocyst and (E) sporulated oocyst with two sporocysts (arrow) and two sporozoites (arrow heads).

1.3 Transmission of infection

N. caninum can be transmitted via either the horizontal or the vertical route (Figure 1.1). Horizontal transmission occurs by the ingestion of tissue cysts containing *N. caninum* bradyzoites, which is relevant for the carnivore definitive host (Dijkstra *et al.*, 2001b), or by the ingestion of food or water contaminated with sporulated oocysts (Dubey *et al.*, 2007). The uptake of sporulated oocysts is the only demonstrated mode of infection after birth in cattle and herbivores in general (McCann *et al.*, 2007). There are no reports of cow to cow transmission of *N. caninum* to date (Dubey *et al.*, 2017). Vertical transmission occurs when tachyzoites from an infected dam cross the placenta and spread to the foetus. Two distinct pathways of vertical transmission, designated exogenous and endogenous transplacental transmission, are possible (Trees and Williams, 2005). Exogenous transplacental transmission occurs when a dam acquires a primary infection following ingestion of *N. caninum* oocysts during pregnancy. Endogenous transplacental transmission is due to the recrudescence of the parasite in a persistently infected dam following the reactivation of bradyzoites into tachyzoites which leads to foetal infection (Williams *et al.*, 2009).

N. caninum is considered as one of the most efficiently transplacentally transmitted organisms. A mother to foetus transmission probability of up to 94.8% was observed in naturally infected cows with high antibody titres (More *et al.*, 2009).

Vertical transmission may lead to abortion; however, in most cases, results in the birth of healthy but congenitally infected calves. These congenitally infected calves are persistently infected and may transmit the parasite to their offspring in consecutive pregnancies, contributing significantly to the maintenance and spread of the infection within the herd (Piergili Fioretti *et al.*, 2003).

Although congenital transmission is considered the major route of infection in cattle, mathematical modelling studies have indicated that it would not be sufficient to sustain the infection within a herd. Therefore, postnatal transmission appears to be required for the disease to become and persist as endemic in a population (French *et al.*, 1999). Although possible, venereal transmission through contaminated semen is considered unlikely. *N. caninum* has been detected in the semen of bulls (Ortega-Mora *et al.*, 2003,

Serrano-Martinez *et al.*, 2007b); however, very high doses of tachyzoites were required for the intra-vaginal infection of cows (Serrano-Martinez *et al.*, 2007c). Despite the detection of *N. caninum* DNA in milk and colostrum of infected cows (Moskwa *et al.*, 2007) and the infection of neonatal calves by feeding milk experimentally contaminated with *N. caninum* tachyzoites (Uggla *et al.*, 1998), there is no evidence that lactogenic transmission can occur in field conditions (Davison *et al.*, 2001, Dijkstra *et al.*, 2001b).

1.4 Molecular and cell biology

1.4.1 *N. caninum* genome and transcriptome

The whole nuclear genome sequence of the *N. caninum* Liverpool isolate (Nc-Liv) was firstly published in 2012 (Reid *et al.*, 2012). Genomic data already available for *T. gondii* (strain ME49) (Gajria *et al.*, 2008), were used as a framework to construct the *N. caninum* genome based on predicted protein sequence similarity. According to early estimations, the nuclear genome of *N. caninum* consists of approximately 61 Mb with 7,121 genes organised in 14 chromosomes (Reid *et al.*, 2012). However, the complete sequence, available in GeneDB <http://www.genedb.org/Homepage/Ncaninum> and ToxoDB <http://www.toxodb.org/toxo/>, is subjected to frequent updates and amendments occurring whenever improved gene annotation data become available. For example, the combination of proteomic data and transcriptomic information generated using the RNASeq technology has recently enabled the identification of *loci* which were not previously annotated (Krishna *et al.*, 2015).

The information currently accessible is restricted to the nuclear genome. DNA sequences contained within the mitochondrion and the apicoplast (i.e. the vestigial non-photosynthetic plastid found in most apicomplexan) of *N. caninum* have not been sequenced to date (Goodswen *et al.*, 2013).

Differences and similarities between the genomes of *T. gondii* and *N. caninum* have been extensively investigated to identify the genetic basis of host restriction, transmission strategies, virulence and zoonotic potential of the two apicomplexans (Ramaprasad *et al.*, 2015). Compared to other eukaryotic organisms, the genomes of

both *T. gondii* and *N. caninum* are characterised by uniquely long (810-860 nucleotides) untranslated regions (UTRs) which enrich most gene families (Ramaprasad *et al.*, 2015). UTRs play a key role in the post-transcriptional regulation of gene expression and in the modulation of mRNA transport from the nucleus, as well as their subcellular localisation, translation efficiency and stability (Mignone *et al.*, 2002). In Apicomplexan parasites with complex life cycles, such as *T. gondii* and *N. caninum*, which require high precision regulation of gene expression, UTRs may play an important post-transcriptional regulatory role within several gene families (Ramaprasad *et al.*, 2015).

Although the genomes of *N. caninum* and *T. gondii* are very similar, with widely conserved regions and a high degree of synteny (i.e. the co-localisation of genes within a chromosome), remarkable differences have been observed (Reid *et al.*, 2012). Such differences are more significant within gene families involved in the host-parasite interactions (Reid *et al.*, 2012, Ramaprasad *et al.*, 2015).

Gene families encoding surface antigens are considerably expanded in *N. caninum*. In particular, the parasite displays a wider set of genes encoding surface-associated glycoprotein denominated SAG-related surface sequences (SRS) (Adomako-Ankomah *et al.*, 2014). However, compared to *N. caninum*, *T. gondii* expresses a greater number of these genes (Reid *et al.*, 2012).

Given the pivotal role of the SRS proteins in host cell recognition and invasion, it was hypothesised that the potentially more restricted host spectrum of *N. caninum* may be linked to differences in the repertoire of surface antigens genes and expression patterns (Nolan *et al.*, 2015, Reid *et al.*, 2012). Nevertheless, further studies are required to support this hypothesis.

Interestingly, genes encoding SAG-unrelated surface antigens (SUSA) which are thought to interact with the host immune system (Pollard *et al.*, 2008) are also more numerous in *N. caninum* although a greater number is expressed in *T. gondii* (Reid *et al.*, 2012).

The repertoires and expression profiles of gene products known or predicted to be localised at the level of the apical complex differ between *N. caninum* and *T. gondii*. For example, the genome of *N. caninum* differs substantially from the genome of *T. gondii* at several *loci* in which genes encoding for virulence factors (rhoptry genes:

ROP18, *ROP16* and *ROP5*) are located (Reid *et al.*, 2012). In addition, two genes encoding proteins of the microneme apical organelles, denominated *MIC26* and *MIC19*, have been identified uniquely in *N. caninum*. Interspecific differences also exist regarding the dense granule genes *GRA11* and *GRA12*, which have been identified in *T. gondii*, are absent from the *N. caninum* genome sequence (Reid *et al.*, 2012).

Recently, the transcriptome analyses of different *N. caninum* isolates, carried out using high throughput RNA sequencing (RNA-Seq), highlighted marked differences between the expression profiles of the high virulence isolate Nc-Spain7 and the low virulence isolate Nc-Spain1H, thus providing useful insights into the mechanisms involved in virulence (Horcajo *et al.*, 2018). When examined throughout the tachyzoite lytic cycle, the expression profile of the low virulence isolate was characterised by the over-expression of genes related to early bradyzoite conversion such as NcBAG1 and NcSAG4. This pre-bradyzoite transcriptome of Nc-Spain1H may be linked to its slower growth rate and may explain the lower virulence of the isolate (Horcajo *et al.*, 2018).

1.4.2 Host cell-parasite interactions

The first step of *N. caninum* tachyzoite invasion into the host cell is the establishment of a reversible, low affinity, contact with the cell membrane. This primary contact is mediated by surface antigens constitutively expressed by the parasite (Hemphill *et al.*, 2004). Once adhered, the tachyzoites orientate themselves perpendicularly to the cell membrane. At this stage, secretory components are sequentially discharged by tachyzoite organelles the micronemes, rhoptries and dense granules (English *et al.*, 2015).

Microneme (MIC) and rhoptry (ROP) proteins contribute to the establishment of a moving junction: a structure which tightly connects the apical pole of the parasite to the plasma membrane and slides over the parasite as it enters the host cell (Besteiro *et al.*, 2011).

Similar to *T. gondii*, it is assumed that *N. caninum* penetrates the host cells by pulling MIC complexes and the moving junction towards its posterior end. In such manner, the tachyzoite invaginates the host cell membrane creating a large vacuole known as

the parasitophorous vacuole (PV) (Nolan *et al.*, 2015). Subsequently, dense granule proteins (GRA) are released into the PV (Ellis *et al.*, 2000, Lally *et al.*, 1997). Although the precise function of GRA proteins is still unknown, they are thought to maintain the environment of the PV enabling survival and multiplication of the protozoa (Liu *et al.*, 2013).

The PV is surrounded by the parasitophorous vacuole membrane (PVM) within which the parasite proliferates by endodyogeny and interacts with many host cell structures and organelles by diverting them to its PV (Hemphill *et al.*, 2006, Nolan *et al.*, 2015). This enables *N. caninum* to sequester and scavenge nutrients from the host cell. These include cholesterol from endocytic organelles and sphingolipids from the Golgi apparatus (Nolan *et al.*, 2015).

When a critical mass is reached, the tachyzoites egress from the host cell (Hemphill 1999). Egression enables the parasite to spread and invade neighbouring cells and results in cell lysis and tissue damage with deleterious consequences for the host (Hemphill *et al.*, 2004).

During the stage conversion from tachyzoite to bradyzoite, the PVM undergoes structural modifications and thickens forming the tissue cyst wall; however, the mechanisms underlying this phenomenon are not completely known (Guionaud *et al.*, 2010).

1.4.3 Antigenic repertoire of *N. caninum*

Most of the *N. caninum* antigens that have been identified and characterised to date, are either localised on the protozoa surface or within the secretory organelles (Vonlaufen *et al.*, 2004). Updated information regarding the proteome of *N. caninum* was recently obtained using high throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS), which enabled the study of the relative abundance of several antigenic proteins in different isolates (Horcajo *et al.*, 2018).

1.4.3.1 Surface antigens: SAG-related sequences (SRS)

The surface of *N. caninum* is covered with glycosylphosphatidylinositol (GPI)-anchored proteins, most of which are members of the SRS superfamily (Lekutis *et al.*, 2001).

Several SRS proteins of *N. caninum* have been identified and characterised to date. These include NcSAG1 and NcSRS2 which are tachyzoite-specific and NcSAG4 (Fernandez-Garcia *et al.*, 2006), NcBSR4 (Risco-Castillo *et al.*, 2007) and NcSRS9 (Risco-Castillo *et al.*, 2011) which are expressed by the bradyzoite stage. In addition, NcSRS67, a SRS with no orthologue in *T. gondii*, has been recently identified in *N. caninum* (Nc-1 strain) tachyzoite extract (Bezerra *et al.*, 2017, Pollo-Oliveira *et al.*, 2013).

In vitro immunolabelling and confocal laser microscopy of *N. caninum* tachyzoites demonstrated the primary role of the two major immunodominant surface antigens, NcSAG1 and NcSRS2 (Howe *et al.*, 1998), within the invasion process (Nishikawa *et al.*, 2000). Monoclonal and polyclonal antibodies directed against these two proteins prevented the tachyzoites from adhering and invading target cells (Haldorson *et al.*, 2006, Nishikawa *et al.*, 2000).

Characterised by high immunogenicity, NcSAG1 and NcSRS2 have been extensively investigated within the development of vaccines and frequently employed for the development of diagnostic tests (Baszler *et al.*, 2008, Otsuki *et al.*, 2013).

1.4.3.2 Microneme proteins (MIC)

Micronemes are cigar-shaped organelles that cluster at the apical end of the parasite (Carruthers and Tomley 2008) and secrete MICs which are not only essential for the adhesion to the host cells but are also crucial for the gliding motility (Dowse and Soldati 2004). MICs are characterised by adhesive domains which interact with receptors localised on the surfaces of target host cells. MICs operate as protein complexes by interacting with several elements such as rhoptry neck proteins (RONs) amongst other. Many MICs have been discovered in *T. gondii* and several *N. caninum* homologues and orthologues have been identified and characterised to date. These include: NcMIC1 (Keller *et al.*, 2002), NcMIC2, NcMIC2-like, NcMIC3 (Naguleswaran *et al.*, 2001), NcMIC4 (Keller *et al.*, 2004) NcMIC6 (Li *et al.*, 2015), NcMIC8 (Wang *et al.*, 2017), NcMIC10 (Yin *et al.*, 2012), SPATR, NcPDI and NcSUB1 which is characterised by proteolytic activity and high immunogenicity (Ybanez *et al.*, 2013).

1.4.3.3 Rhoptry proteins (RONs and ROPs)

Rhoptries are club-shaped organelles located at the anterior pole of the apicomplexans (Kemp *et al.*, 2013). Rhoptry proteins comprise the rhoptry neck proteins (RONs), which are localised in long neck-like part of the organelle and are involved in the formation of the moving junction (Marugan-Hernandez *et al.*, 2011), and the ROP kinase family: a group of proteins implicated as determinants of virulence in both *N. caninum* and *T. gondii* (Reid *et al.*, 2012).

A NcROP5-deficient strain of *N. caninum* which was experimentally constructed, exhibited significantly weaker cell invasion capabilities, reduced proliferation and virulence compared to the Nc-1 wild type strain suggesting a key role of this protein within cell invasion mechanisms (Ma *et al.*, 2017).

1.4.3.4 Dense granule proteins (GRA)

GRA proteins are secreted by vesicles called dense granules which are found throughout the parasite but generally concentrated at the apical end. Despite fifteen GRA proteins have been identified in *N. caninum*, either at the protein or transcriptional levels (Reid *et al.*, 2012), only few have been characterised to date. These include NcGRA1 (Atkinson *et al.*, 2001), NcGRA2 (Ellis *et al.*, 2000), NcGRA6 (Lally *et al.*, 1996b), NcGRA7 (Alvarez-Garcia *et al.*, 2007, Hemphill *et al.*, 1998) and NcMAG1 (Guionaud *et al.*, 2010) among others.

NcGRA2 and NcGRA7 are abundantly expressed by *N. caninum* tachyzoites and secreted during and after invasion into the parasitophorous vacuole (Vonlaufen *et al.*, 2004). Importantly, NcGRA7 has been successfully used for the development of tests for the detection of specific antibodies in sera from infected cattle (Aguado-Martinez *et al.*, 2008).

Furthermore, like *T. gondii*, *N. caninum* expresses a type-I nucleoside triphosphate hydrolase (NTPase) which is also localised within the dense granules (Mercier and Cesbron-Delauw 2015). The upregulation of NcNTPase observed during tachyzoite egression from the host cell suggested a potential role of this protein in the progression of the lytic cycle of the parasite (Pastor-Fernandez *et al.*, 2016).

1.4.4 Stage-specific expression of *N. caninum* antigens

N. caninum is characterised by differential protein expression between tachyzoites and bradyzoites (Kang *et al.*, 2008b) depending on variations in gene transcription between the two parasite stages (Kang *et al.*, 2008a). Similar to *T. gondii*, *N. caninum* tachyzoites and bradyzoites can be differentiated by transmission electron microscopy (TEM), based on differences in the ultrastructural morphology, or through the detection of antigens that are expressed in a stage-specific fashion (Vonlaufen *et al.*, 2004).

Differential expression of surface antigens, which appears to be a strategy to evade the host immune system, has been observed in most apicomplexan parasites. In *T. gondii*, the major surface antigens TgSAG1 and TgSAG2 are specifically expressed at the tachyzoite stage (Kasper 1989). Likewise, the *N. caninum* immunodominant surface antigens NcSAG1 and NcSRS2 are highly expressed by the tachyzoite stage and down-regulated during the tachyzoites-to-bradyzoite stage conversion (Vonlaufen *et al.*, 2002).

Although highly expressed by the tachyzoite stage, NcGRA7 is also expressed by the bradyzoite stage. The antigen has been shown to be differentially located in the tachyzoite and the bradyzoite stages of *T. gondii* (Torpier *et al.*, 1993) and has been detected at the periphery of the tissue cysts after stage conversion in *N. caninum* (Fuchs *et al.*, 1998, Vonlaufen *et al.*, 2004).

In addition to NcGRA7, other dense granule proteins, such as NcGRA1 and NcGRA2, have been detected within the tissue cyst wall of *N. caninum* using immunofluorescence (Kang *et al.*, 2008a, Kang *et al.*, 2008b). Differences in the expression profiles of these antigens were instrumental in the discrimination between tachyzoites and bradyzoites *in vitro*. In addition, proteomic analysis of *N. caninum* during stage differentiation showed that NcGRA9 is highly expressed by the bradyzoite stage (Marugan-Hernandez *et al.*, 2010). Similarly, TgGRA9, the orthologue of NcGRA9 in *T. gondii*, was abundantly expressed by the bradyzoite stage and efficiently secreted in the cyst wall matrix (Caffaro *et al.*, 2013). However, this antigenic protein is expressed by tachyzoites and bradyzoites in both *N. caninum* (Marugan-Hernandez *et al.*, 2010) and *T. gondii* (Adjogble *et al.*, 2004).

Furthermore, NcMIC1 was not detected in *N. caninum* tissue cysts successfully stained with MAb CC2, a monoclonal antibody specific for a cyst wall-associated antigen. This suggested that the expression of this MIC protein may be significantly reduced during the quiescent stage of the parasite (Keller *et al.*, 2002).

Immunolabelling using either monoclonal or polyclonal antibodies as well as the analysis of cDNA expression libraries has enabled the identification of several bradyzoite-specific antigens (Vonlaufen *et al.*, 2004). These include NcSAG4 (Fernandez-Garcia *et al.*, 2006), NcBSR4 (Risco-Castillo *et al.*, 2007), NcSRS9 (Risco-Castillo *et al.*, 2011), NcSRS13 (Reid *et al.*, 2012), NcMAG1 (Guionaud *et al.*, 2010) and NcBAG1 (Kobayashi *et al.*, 2013).

Several bradyzoite-specific antigens are shared between *N. caninum* and *T. gondii*.

NcSAG4 and NcSRS6 have been shown to be overexpressed in bradyzoites of both *T. gondii* (Wasmuth *et al.*, 2012) and *N. caninum* (Fernandez-Garcia *et al.*, 2006).

Polyclonal antibodies raised against TgBAG1 have been shown to cross-react with *N. caninum* tissue cysts (Tunev *et al.*, 2002), in which NcBAG1 is highly expressed (Kobayashi *et al.*, 2013). However, other antigens which are considered to be tachyzoite-specific in *T. gondii* showed overexpression in *N. caninum* bradyzoites; for example, TgROP9 has been reported as a tachyzoite-specific antigenic protein involved in the early stages of cell invasion in *T. gondii*, whereas its orthologue in *N. caninum* (NcROP9) is highly expressed at the bradyzoite stage (Marugan-Hernandez *et al.*, 2011).

Nevertheless, the wealth of information regarding the antigenic repertoire of the bradyzoite stage is limited. This is likely due to low bradyzoite production levels both *in vivo* (McGuire *et al.*, 1997b) and *in vitro* (Weiss *et al.*, 1999).

In contrast to *T. gondii*, in which sporozoite-specific antigens have been identified (Hill *et al.*, 2011), there is scarce information regarding antigens which might be specifically displayed by the sporozoite stage of *N. caninum*.

1.5 Bovine neosporosis

1.5.1 Pathogenesis

The pathogenesis of bovine neosporosis is complex and is not yet fully elucidated. Regardless of whether the *N. caninum* challenge originates from a primary infection during pregnancy or the reactivation of a persistent quiescent infection, the disease is initiated by a maternal parasitaemia (Macaldowie *et al.*, 2004). This was indicated by the detection of *N. caninum* DNA in the blood of naturally infected cattle (Okeoma *et al.*, 2004). The parasite then establishes itself in the cells of the placenta, invading the caruncular septa in the placentome, from where it is able to cross to and infect trophoblast cells in the chorionic villi (Barr *et al.*, 1994, Bartley *et al.*, 2004, Maley *et al.*, 2003).

Rapid multiplication of the tachyzoites takes place within the placental tissue eliciting a non-suppurative inflammatory response with infiltration of maternal inflammatory cells, mostly CD4⁺, CD8⁺ and $\gamma\delta$ T-cells (Innes *et al.*, 2005). CD4⁺ T cells and $\gamma\delta$ T-cells produce IFN- γ (Innes *et al.*, 2005). Simultaneously with the onset of placental infection, the parasite enters the foetal bloodstream and invades further tissues, with a predilection for the CNS (Macaldowie *et al.*, 2004).

At present, it is not clear whether the foetal damage is due to a primary tissue damage (i.e. necrosis), deriving from the multiplication of *N. caninum* (Gibney *et al.*, 2008), or to insufficient oxygenation and nutrition secondary to placental damage. Experimental evidence suggested that the release of maternal pro-inflammatory cytokines at the maternofetal interface level might play a key role in leading to foetal death (Innes 2007, Rosbottom *et al.*, 2008).

1.5.2 Consequences of the disease

In cattle, neosporosis affects primarily pregnant dams and their foetuses; less frequently congenitally infected new-born calves.

Generally, *N. caninum* naturally infected adult cattle do not show any clinical signs (Dubey and Lindsay 1996); however, transitory fever associated with inappetence has been observed in experimentally infected animals (Dubey *et al.*, 2006a). Nevertheless, two studies carried out in Canada reported reduced growth rates in weaned beef steers

seropositive to the parasite (Barling *et al.*, 2001, Barling *et al.*, 2000). In a later study such a relationship in beef cattle was not observed (Waldner *et al.*, 2004). Reduced milk production, which is correlated with *N. caninum* abortions due to the interference with timing and length of lactation and dry periods, body condition and udder health (McAllister 2016), does not appear to be associated with the presence of *N. caninum*-specific antibodies in non-aborting dairy cows (Bartels *et al.*, 2006b).

Abortion is the main clinical manifestation; however, reabsorption of the *conceptus*, still-birth, the birth of impaired calves or the birth of asymptomatic calves that are persistently infected with the parasite may also occur (Dubey and Lindsay 2006, McAllister 2016). *N. caninum* abortions can occur from 4 months of gestation to term, although they are typically observed within the fifth and seventh month (McAllister 2016).

Several factors contribute to determine the different outcomes that the infection may have: (1) infectious dose, (2) virulence of the *N. caninum* isolate and (3) stage of pregnancy during which infection occurs.

As observed in sheep experimentally infected with *N. caninum*, the induction of disease may be dose-dependent (Buxton *et al.*, 1997). Moreover, it has been shown that some *N. caninum* isolates induce abortion more readily than others (Regidor-Cerrillo *et al.*, 2014).

The timing during pregnancy in which infection occurs is considered particularly important (Williams *et al.*, 2000). Evidence suggested that the gestational age of the foetus when it encounters the parasite, the relative maturity of the foetal immune system, and the effectiveness of the maternal immune responses are all critical factors in determining the clinical outcome (Innes *et al.*, 2001, 2002). This is supported by the increased abortion risk during the second trimester (Lopez-Gatius *et al.*, 2004b, Pare *et al.*, 1997) when the foetus is not yet fully immunocompetent (Osburn 1981). In addition, at this stage the downregulation of the maternal Th1 cellular immune responses, with a bias towards Th2-like responses, appears to be important to enable the dam to successfully carry the pregnancy (Innes *et al.*, 2001).

In general, the earlier the infection occurs in pregnancy, the more severe the consequences to the developing foetus (Macaldowie *et al.*, 2004, Williams *et al.*, 2000). Infection during early pregnancy is likely to pass unnoticed but may result in

the foetus being reabsorbed giving the outward appearance of infertility (Innes 2007). Experimental infection of cattle with *N. caninum* at day 70 of gestation resulted in rapid foetal death (Macaldowie *et al.*, 2004).

On the other hand, the presence of a quiescent infection acquired prior to pregnancy, in persistently infected dams, appears not to affect the early foetal period, despite having a significant abortive effect after the first trimester of gestation (Lopez-Gatius *et al.*, 2004b).

Abortion induced by *N. caninum* can arise from the third month of gestation to term; in most diagnosed cases, infected dams abort at 5 to 6 months of pregnancy (Lopez-Gatius *et al.*, 2004b, Williams *et al.*, 2009). During the second trimester, depending on the extent of placental and foetal lesions, *N. caninum* infection can cause either abortion or the birth of congenitally infected calves (Dubey *et al.*, 1992b, Maley *et al.*, 2003, Rosbottom *et al.*, 2008, Williams *et al.*, 2000). Later in pregnancy, during the third trimester, the parasite generally causes the birth of congenitally infected live calves which show few or no clinical signs, as demonstrated by several experimental studies (Benavides *et al.*, 2012, Gibney *et al.*, 2008, Williams *et al.*, 2000). Similar to the infection in cows post-natally, in these animals, *N. caninum* establishes persistent infections in the CNS. Such persistent infections are characterised by the presence of tissue cysts containing bradyzoites that multiply slowly (Dubey *et al.*, 2017).

Sporadically, clinical signs are observed in congenitally infected calves younger than 2 months of age. Clinically affected animals may be underweight, weak and unable to rise. Flexion or hyperextension of the forelimbs and/or hind limbs, ataxia, decreased spinal reflexes, loss of conscious proprioception, scoliosis, hydrocephalus, spinal cord narrowing and exophthalmia have also been reported (Dubey and Lindsay 2006).

Repeated abortions due to neosporosis are possible but infrequent. A study, examining aborted fetuses collected from dairy herds with and without history of *N. caninum* abortion reported that only 1.5% of the aborting cows aborted twice because of the parasite (Anderson *et al.*, 1995).

1.5.2.1 Abortion patterns

Distinct abortion patterns may occur: (1) epidemic and (2) endemic (sporadic) (McAllister *et al.*, 2000, Yaeger *et al.*, 1994). Epidemic neosporosis is characterised

by abortion storms defined as the abortion in more than 10% of the cattle at risk (i.e. pregnant) within a period of 12 weeks. Conversely, during endemic neosporosis a limited number of cases are observed over several months or years within a herd (Goodswen *et al.*, 2013). The various patterns of abortions may be related to the different routes of transmission. Epidemic patterns are mainly associated to a primary horizontal (point source) infection in a group of naïve animals (Basso *et al.*, 2010), whereas endemic patterns occur as a result of recurrent vertical transmission within infected family lines (reviewed by Dubey *et al.*, 2007).

1.6 Immune responses to *N. caninum* in cattle

Following exposure to *N. caninum*, cattle develop cell-mediated and humoral immune responses which provide only partial protection against the parasite.

Clearance of the parasite from infected cattle is considered unlikely, if not impossible, and infected cows have a greater risk of abortion compared to non-infected (Lopez-Gatius *et al.*, 2004a, Weston *et al.*, 2005). Despite this, a degree of protection against disease is indicated by the fact that previously infected dams are less likely to abort than naïve animals as observed in a case of a point source exposure to *N. caninum* (McAllister *et al.*, 2000). In addition, there is evidence that the risk of abortion and vertical transmission decreases during subsequent pregnancies (Dijkstra *et al.*, 2003). A vaccination study reported that cows subcutaneously inoculated prior to mating and subsequently challenged at mid-gestation developed immune responses sufficient to prevent vertical transmission to their offspring (Innes *et al.*, 2001). In another vaccination experiment, the intravenous injection with live *N. caninum* tachyzoites, but not whole-tachyzoite lysate, proved effective in protecting cattle against foetopathy (Williams *et al.*, 2007).

Given the obligate intracellular nature of *N. caninum*, cell-mediated immune responses are expected to play a major role in controlling the infection and reducing the multiplication of the parasite in the host (Innes *et al.*, 2005). This is supported by the positive correlation between protection against foetal death and the detection of strong

cell-mediated and gamma interferon (IFN- γ) responses detected in cows immunised and subsequently challenged with live tachyzoites (Williams *et al.*, 2007).

The contribution of *N. caninum*-specific antibodies to protective immunity is unclear (Andrianarivo *et al.*, 2001); however, a complementary role of the humoral immune responses in reducing parasite invasion of host cells has been suggested (Haldorson *et al.*, 2006, Innes *et al.*, 2002).

1.6.1 Cell-mediated immune responses

As for other intracellular pathogens, the host immune responses to *N. caninum* are generally characterised by a T-helper type 1 (Th1) cell-mediated immune response, in which the activation of antigen presenting cells (APCs) triggers the production of specific cytokines such as interleukin-12 (IL-12) that activates T-cells (Dion *et al.*, 2011).

Polymorphonuclear neutrophils (PMNs) may contribute significantly to the early innate host defences occurring during *N. caninum* infection (Villagra-Blanco *et al.*, 2017). Effector mechanisms used by PMNs include phagocytosis, production of reactive oxygen species (ROS), release of antimicrobial peptides and synthesis of neutrophil extracellular traps (NETs) (Brinkman *et al.*, 2004). NETs are web-like structures consisting of granule and cytosolic proteins assembled on a scaffold of decondensed chromatin (Brinkman *et al.*, 2004, Papayannopoulos, 2017) which trap, neutralise and kill bacteria (Brinkman *et al.*, 2004), fungi (Urban *et al.*, 2006), viruses (Saitoh *et al.*, 2012) and protozoan parasites (Abi Abdallah *et al.*, 2012). *In vitro*, *N. caninum* tachyzoites have been shown to trigger the formation of NETs, preventing the penetration of the parasite into bovine umbilical vein endothelial cells (BUVEC) (Villagra-Blanco *et al.*, 2017). These findings suggested that NETs may represent an effective mechanism during the early innate immune response, which might contribute to a reduction in infection rates during the acute phase of bovine neosporosis (Villagra-Blanco *et al.*, 2017).

The early innate immune response to *N. caninum* infection is mediated by activated natural killer (NK) cells (Boysen *et al.*, 2006). NK cells are responsible for the production of cytokines and the lysis of target cells (Carayannopoulos and Yokoyama, 2004, Korbel *et al.*, 2004). The cytotoxicity of NK cells, induced by the release of IL-

IL-12 from activated APCs, plays a key role in the early stages of protozoan infections, before the onset of T and B cell-mediated immune responses (Korbel *et al.*, 2004). Intact *N. caninum* tachyzoites directly trigger bovine NK cells to produce IFN- γ *in vitro*, even in the absence of IL-12 (Boysen *et al.*, 2006).

During *N. caninum* infections, NKs are considered important for providing the cytokine environment necessary for the activation of CD4⁺ and CD8⁺ T cells which are responsible for the recognition and destruction of parasitised cells (Klevar *et al.*, 2007).

Activated, CD4⁺ and CD8⁺ T cells produce pro-inflammatory cytokines such as IFN- γ , TNF α , IL-12 and IL-17 which initiate an inflammatory response. The activation of cytotoxic CD4⁺ T cells and the production of IFN- γ play a particularly important role in the control of *N. caninum* (Boysen *et al.*, 2006, Rojo-Montejo *et al.*, 2013, Rosbottom *et al.*, 2007, Staska *et al.*, 2005).

In vitro, IFN- γ and TNF- α demonstrated an inhibitory effect on the intracellular multiplication of the parasite (Yamane *et al.*, 2000). Furthermore, *N. caninum*-specific CD4⁺ T cell clones and NK cells can efficiently lyse cells infected with the parasite (Boysen *et al.*, 2006, Staska *et al.*, 2003, Yamane *et al.*, 2000).

High systemic levels of IFN- γ have been correlated with protection against *N. caninum* abortion in both experimentally (Williams *et al.*, 2007) and naturally infected cattle (Lopez-Gatius *et al.*, 2007). Significantly higher antigen-specific lymphocyte proliferation and IFN- γ production in PBMCs and lymph node tissue were observed in non-aborting compared to aborting dams following subcutaneous challenge with *N. caninum* (NC-1 strain) at mid-gestation (day 140) (Bartley *et al.*, 2013a).

An *in vivo* study suggested that the presence of a parasite-induced maternal immune response in the placenta, consisting of CD4⁺ and CD8⁺ T cells infiltration with significant up-regulation of the expression of IFN- γ , IL-4, IL-10, IL-12 and TNF- α , is not detrimental to foetal survival, but might contribute to the control of *N. caninum* at the placental level (Rosbottom *et al.*, 2011).

In a recent study, PBMCs and mononuclear cells from the spleen and uterine lymph nodes of non-aborting heifers, experimentally infected (Nc-Spain 7 strain) at 110 days of pregnancy, showed lower *in vitro* IFN- γ /IL-4 production ratios and higher IL-4 production compared to dams in which foetal mortality occurred. These findings

highlighted that the protective role might not be exclusively dependant on the synthesis of IFN- γ (Darwich *et al.*, 2016).

Although the pro-inflammatory response is beneficial for the elimination of the parasite, in the gravid uterus it may have a detrimental effect (Innes 2007). At the placental level, inflammatory responses with the production of cytokines, chemokines, NO and prostaglandins (Buxton *et al.*, 2002, Dubey *et al.*, 2006b, Quinn *et al.*, 2002a) may result in tissue damage leading to loss of placental functionality with insufficient oxygenation and vascular supply of nutrients which may ultimately lead to abortion (Canton *et al.*, 2014, Macaldowie *et al.*, 2004). Given the protective effect of the Th1 pro-inflammatory responses with production of IFN- γ observed in many studies, it has been postulated that excess of IFN- γ response over a critical threshold would determine the onset of foetopathy (Almeria *et al.*, 2016).

In pregnant cattle, the outcome of *N. caninum* infection (i.e. abortion or foetal infection without clinical signs) is dependent on the balance between Th1 type responses which mediate the control of the parasite and Th2 type responses which occur physiologically to enable successful foetal implantation (Innes *et al.*, 2005). During pregnancy, the rise in progesterone levels inhibits the Th1 responses, which results in the dose-dependent inhibition of the expression of IFN- γ and IL-17, and promotes the Th2 responses (Maeda *et al.*, 2013). The Th2 immune response mediates the activation and maintenance of humoral responses and is characterised by the production of anti-inflammatory cytokines, such as IL-10, IL-4 and transforming growth factor beta (TGF β) which counter-regulate the pro-inflammatory Th1 response (Almeria *et al.*, 2017). These mediators are highly expressed at the maternofetal interface and are involved in the regulation of pregnancy avoiding foetal rejection (Entrican 2002). At the same time, the natural bias toward Th2 type responses may alter the host-parasite interaction thus enabling the protozoan to become more active in the pregnant host facilitating vertical transmission (Innes *et al.*, 2002).

Recently, the reproductive immunology Th1/Th2 paradigm of women has been extended into the Th1/Th2/Th17 paradigm. Th17 cells produce pro-inflammatory cytokines IL-17, IL-21 and IL-22 which are involved in the host defence against pathogens and the development of autoimmune diseases (Saito *et al.*, 2010). The

downregulation of Th17 responses may be important to enable successful embryo implantation and pregnancy (Saito *et al.*, 2010).

In cattle, Th17 cell responses with the production of IL-17 have been identified and a potential protective role against foetal death and host tissue damage during neosporosis has been suggested (Peckham *et al.*, 2014). It has been hypothesised that this role might be related to the production of protective antibodies or the involvement of Th17 cells in the orchestration of neutrophil/effector cell influx at the site of infection; however, further investigations are required to elucidate the role of this type of cell-mediated immune response during neosporosis (Peckham *et al.*, 2014).

In addition to CD4⁺ T cells, $\gamma\delta$ -T cells have been identified as a major source of IL-17 during the infection with protozoan parasites, such as *Plasmodium berghei* (Ribot *et al.*, 2010) and *Leishmania major* (Akilov *et al.*, 2009). A role of $\gamma\delta$ -T cells in the production of IL-17 during *N. caninum* infection has been observed. However, these cells may act as a shorter term innate immune response compared to Th17 cells, which would provide more consistent IL-17 production (Peckham *et al.*, 2014).

1.6.2 Humoral immune responses

Antibody responses to *N. caninum* are characterised by the early onset of IgM antibodies which peak two weeks post-infection and decline rapidly within four weeks (De Marez *et al.*, 1999). Whereas, the concentrations of IgG antibodies can increase for 3-6 months and are believed to persist for life after a primary infection. Subcutaneous or intravenous inoculation with *N. caninum* tachyzoites elicit the production of specific antibodies within 14 days (Bartley *et al.*, 2004, Maley *et al.*, 2001). An initial increase in the levels of the IgG₁ subclass is followed by a slightly delayed rise in IgG₂ (Williams *et al.*, 2000).

Serum antibody responses to *N. caninum* fluctuate throughout gestation depending on the physiological status of the dam (Andrianarivo *et al.*, 2005, Guy *et al.*, 2001) and the activity of the parasite (Innes 2007). A marked increase in antibody titres is observed during the second half of gestation and has been associated with vertical transmission in numerous experimental and field studies (Guy *et al.*, 2001, Nogareda *et al.*, 2007, Pare *et al.*, 1996, Stenlund *et al.*, 1999). The rise of maternal antibody levels, mainly IgG₂, may be a consequence of the reactivation of bradyzoites into

tachyzoites in persistently infected animals (Guy *et al.*, 2001). Another longitudinal study in non-aborting cows showed variations in *N. caninum*-specific antibody levels during different gestational periods, with a significant increase in antibody titres, after the fifth month of gestation (Cardoso *et al.*, 2009). A correlation between the timing of an increase in antibodies titres and the risk of congenital infection was observed (Williams *et al.*, 2003, Pare *et al.*, 1997).

1.6.3 Foetal immune responses

Given the importance of the gestational age in determining the outcome of *N. caninum* infection, foetal immune responses represent a critical factor in the pathogenesis of bovine neosporosis (Innes *et al.*, 2002).

During gestation, bovine foetuses progressively develop immunocompetence. Mitogenic cellular immune responses in foetal splenic and thymic cells were observed around day 100 of pregnancy in *N. caninum*-infected bovines (Innes *et al.*, 2005). From day 120 the foetus can mount specific cell-mediated and humoral immune responses to the parasite (Almeria *et al.*, 2003, Bartley *et al.*, 2004). As a consequence, infections occurring before this gestational age (during the first trimester) are likely to find the foetus unable to respond effectively to the pathogen which can rapidly proliferate throughout the foetal tissues (Innes 2007) causing foetal death and reabsorption (Collantes-Fernandez *et al.*, 2006, Regidor-Cerrillo *et al.*, 2014).

The stage of foetal development plays a key role in the outcome of other reproductive infections of cattle. Maternal infection with Akabane virus can cause abortions, stillbirths, premature births and congenital defects, whose type is dependent on the stage of pregnancy. In a prospective study, infections between 76 and 104 days of gestation resulted in foetal hydranencephaly and porencephaly, whereas cases of arthrogryposis were observed as a consequence of infections between 103 and 174 days of pregnancy (Kirkland *et al.*, 1988). Foetuses of less than 2 months of age were not affected by the virus (Kirkland *et al.*, 1988).

In analogy with the *in utero* infection with bovine viral diarrhoea virus (BVDV), the occurrence of immunotolerance in calves congenitally infected with *N. caninum* has been hypothesised. Before day 120 of pregnancy, transplacental infections with the non-cytopathic (ncp) biotype of BVDV can result in the birth of persistently infected

calves which do not produce any immune responses to the pathogen (i.e. they are immunologically tolerant) (Anderson *et al.*, 2000a, McInnes *et al.*, 2006b).

At present, there is not sufficient evidence that this may also occur during *N. caninum* infections and this scenario is considered likely to be infrequent although possible.

With the development of specific foetal immune responses around mid-gestation (Bartley *et al.*, 2012), the outcome of infection at this stage is variable with the possibility of the birth of congenitally infected asymptomatic calves or the occurrence of abortion. At this stage, foetal death is likely due to the incomplete development of the innate immune responses mediated by phagocytic cells which do not fully develop until late gestation (Horcajo *et al.*, 2016).

Infections occurring in late gestation are more likely to result in the birth of live congenitally infected calves that do not show any obvious clinical signs (Collantes-Fernandez *et al.*, 2006, Rosbottom *et al.*, 2007).

1.7 Diagnosis of bovine neosporosis

Although epidemiological data and clinical history, such as the abortion pattern within the herd and the gestational age at the time of abortion, may be suggestive of neosporosis, laboratory methods are required for a definitive diagnosis (McAllister 2016).

From the clinician's point of view, it is important to differentiate between the recognition of *N. caninum* as the cause of abortion and the identification of animals infected with the protozoan. Being able to discriminate between infected and uninfected animals is the basis of disease management and a number of diagnostic tools have been developed for this purpose (Dubey and Schares 2006).

Since the direct detection of *N. caninum* in aborted fetuses does not necessarily rule out other causes of abortion, accurate analysis of the severity of the lesions and exclusion of other causes is required. Therefore, the diagnosis of bovine neosporosis should be approached within a wider diagnostic plan of abortion in which the major causes of foetal mortality in cattle should be included (Dubey *et al.*, 2007).

In some cases, diagnosis of bovine neosporosis can only be made at the herd level by combining the information obtained by examining aborted fetuses with maternal

serology and by comparing the presence of *N. caninum*-specific antibodies in aborting and non-aborting animals. A statistically significant increase of the abortion risk in seropositive cows would be suggestive of an ongoing bovine neosporosis problem (McAllister 2016).

Examination of aborted fetuses and serological testing should complement each other where possible.

1.7.1 Examination of aborted fetuses

The direct examination of aborted fetuses offers the greatest chances of reaching an aetiological diagnosis regardless the cause of bovine abortion (McAllister 2016).

Gross lesions are rare in *N. caninum*-associated abortions, although hydrocephalus, hypoplasia of the cerebellum and medulla as well as scattered pale to dark *foci* of necrosis in the brain, spinal cord, heart and skeletal muscle have been reported (Dubey *et al.*, 1998, Dubey and Schares 2006).

Entire chilled fetuses and placental cotyledons, or tissue samples, can be submitted to the veterinary diagnostic centres for pathological, histopathological, serological and molecular analyses.

1.7.1.1 Histopathology

Microscopic lesions are generally localised in the CNS, heart, liver, and placenta. Distinctive histological findings are multifocal non-suppurative encephalomyelitis with mononuclear perivascular cuffs, microgliosis and necrosis (Helman *et al.*, 1998, Nishimura *et al.*, 2013a), which is considered almost pathognomonic (Dubey 2003, Dubey and Schares 2006, Morales *et al.*, 2001). Non-suppurative myocarditis, myositis and periportal hepatitis with necrosis have also been described (Dubey and Schares 2006).

At the placental level, the lesions are typically restricted to the cotyledons in which focal areas of necrosis and inflammation with mononuclear infiltrate may be observed (Piergili Fioretti *et al.*, 2003).

1.7.1.2 Immunohistochemistry

Immunohistochemistry (IHC), employing monoclonal (MAbs) or polyclonal antibodies recognising *N. caninum* tachyzoites or bradyzoite antigens, is frequently used alongside the analysis of histopathological lesions to improve the diagnosis (Uzeda *et al.*, 2013). This technique has the advantage of enabling the assessment of the number, distribution and micro-anatomical localisation of pathogens in tissue sections (Ramos-Vara *et al.*, 2008). IHC is particularly useful when characteristic lesions of neosporosis are not evident (Boger and Hattel 2003).

Cross-reactivity between *N. caninum* and *T. gondii* has been observed in tissues of both experimentally and naturally infected animals examined with IHC (reviewed in Gondim *et al.*, 2017). However, in one study, the combination of two MAbs targeting NcSRS2 and NcGRA7 enabled the specific detection of *N. caninum* by avoiding cross-reactions with related apicomplexan in tissue sections from bovine foetuses (Uzeda *et al.*, 2013). Importantly, other genetically related protozoan, such as *Sarcocystis* spp. and *Besnoitia besnoiti*, should be considered when validating the specificity of novel IHC protocols due to the possibility of cross-reactions (Uzeda *et al.*, 2013).

1.7.1.3 Molecular detection of *N. caninum*

More recently, polymerase chain reaction (PCR) has become increasingly applied to the direct detection of *N. caninum* DNA (or RNA) in fresh, frozen, formalin-fixed and paraffin wax-embedded foetal tissues (Dubey and Schares 2006). Foetal brain is generally considered the best specimen for PCR analysis followed by heart, liver and placenta (Dubey 2003). Nevertheless, protocols for the amplification of parasite DNA from different samples such as cerebrospinal fluid (CSF) (Buxton *et al.*, 2001) and amniotic fluid (Ho *et al.*, 1997) have been described.

Due to its analytical sensitivity, PCR is particularly helpful for the diagnosis of bovine abortions when foetal and placental tissues have undergone autolysis and histopathology would not be feasible (McAllister 2016).

The likelihood of detecting *N. caninum* in specific foetal tissues may vary depending on the gestational stage at which foetal death occurs. In one study, the frequency of PCR positive results in brain, heart, kidney and lung samples was significantly higher in foetuses aborted during the first trimester compared to foetuses aborted during the

second and third (Collantes-Fernandez *et al.*, 2006). Interestingly, in foetuses aborted during late gestation (third trimester) *N. caninum* DNA was almost exclusively detected in the foetal brain and only sporadically in diaphragm, heart and kidney. These findings were attributed to variations of the parasite load in certain foetal tissues during pregnancy (Collantes-Fernandez *et al.*, 2006) and are also likely to reflect the development of the foetal immune response during gestation.

A variety of PCR protocols have been established. These include conventional (Holmdahl and Mattsson 1996), nested (Buxton *et al.*, 1998) and multiplex PCRs (reviewed in Dubey *et al.*, 2017). Quantitative PCRs have also been developed and have the advantage to enable the estimation of the parasitic burden in tissue of aborted foetuses (Nishimura *et al.*, 2013a, Regidor-Cerrillo *et al.*, 2014, Reitt *et al.*, 2007).

The target DNA sequences reported encompass: the internal transcribe spacer 1 (ITS1) region (Buxton *et al.*, 1998, Holmdahl and Mattsson 1996), the Nc-5 gene (Liddell *et al.*, 1999), the 18S and 28S ribosomal DNA (rDNA) (Fazaeli *et al.*, 2000), the HSP70 gene and the 14-3-3 gene (Lally *et al.*, 1996a). Amongst these ITS1 and Nc-5 are the most frequently cited in literature (reviewed in Sinnott *et al.*, 2017).

Furthermore, PCR methods are key tools for the study of *N. caninum* genetic heterogeneity through the analysis of variable genetic *loci*, such as micro- and mini-satellites, which can provide additional epidemiological and phylogenetic information (Campero *et al.*, 2015, Medina-Esparza *et al.*, 2016).

Importantly, with the current availability of the sequenced genome of *N. caninum* Liverpool (Nc-Liv) the development of further PCR protocols using novel DNA targets would be enormously facilitated (Goodswen *et al.*, 2013).

1.7.1.4 Antibody detection in foetal fluids

Beyond the stage of foetal immunocompetence (from 110-120 days of gestation) (Swift and Kennedy 1972), many but not all foetuses develop specific antibodies in response to the transplacental invasion of *N. caninum* tachyzoites (Alvarez-Garcia *et al.*, 2003, Bartley *et al.*, 2004). Such antibody responses can be detected in foetal blood, pleural and peritoneal serosanguinous fluids as well as in the abomasal content (Dubey and Schares 2006). The immunoglobulins detected are predominantly IgG₁, although IgG₂ may also be detected (Andrianarivo *et al.*, 2001).

The sensitivity of foetal serology, assessed with either IFAT or ELISA, has been reported to be low (Alvarez-Garcia *et al.*, 2003, Pereira-Bueno *et al.*, 2003, Sondgen *et al.*, 2001). In one study, only 25% of the foetuses with histopathological lesions suggestive of neosporosis were also antibody positive (Pereira-Bueno *et al.*, 2003). Consequently, negative foetal serology results do not necessarily rule out *N. caninum* as the causative agent. Reduced sensitivity may result from the lack of foetal immunocompetence or the degradation of the immunoglobulins during the autolytic processes which follow the death and expulsion of the aborted foetus (Wouda *et al.*, 1997). Nevertheless, the demonstration of antibodies against *N. caninum* in foetal fluids represents the first specific indicator of the involvement of *N. caninum* in foetal infection (De Meerschman *et al.*, 2002).

Alongside the aborted foetus and placenta, the submission of a serum sample from the aborting dam for serological analysis is highly recommended within a comprehensive diagnostic strategy for bovine neosporosis and bovine abortions in general (McAllister 2016).

1.7.2 *In vivo* diagnosis of bovine neosporosis

The determination of specific antibodies in cattle serum, plasma or milk is indicative of *N. caninum* infection (Dubey 2003), and is currently considered the primary method for the diagnosis of bovine neosporosis in live animals (Alvarez-Garcia *et al.*, 2013). Although the PCR detection of *N. caninum* DNA in blood (Ferre *et al.*, 2005, Okeoma *et al.*, 2004), serum (McInnes *et al.*, 2006b), semen (Ferre *et al.*, 2005, Ortega-Mora *et al.*, 2003, Serrano-Martinez *et al.*, 2007b), colostrum and milk (Moskwa *et al.*, 2007) as well as the analysis of pro-inflammatory cytokines as indicators of exposure to the parasite (reviewed in Almeria and Lopez-Gatius 2015) could also be applied *in vivo*, these markers of infection are confined to the research field because of their transitory nature.

1.7.2.1 Serological techniques

Numerous techniques have been developed for the detection of *N. caninum*-specific antibodies. These encompass a wide variety of enzyme-linked immunosorbent assays (ELISAs), along with the indirect immunofluorescent antibody test (IFAT), *Neospora*

agglutination test (NAT), Western blot (WB) analysis and immunochromatographic test (Sinnott *et al.*, 2017).

1.7.2.1.1 ELISA

Routinely, the ELISA is the elective technique for the analysis of bovine sera within the investigation of bovine abortion outbreaks and for the serological screening of cattle herds as it enables high throughput analyses.

Numerous commercial and in-house ELISAs, designed on different formats (e.g. indirect, competitive, sandwich or dot ELISA), are available. These assays utilise a variety of antigen preparations such as whole or fixed *N. caninum* tachyzoites, aqueous or detergent soluble tachyzoite extracts, single native or recombinant tachyzoite antigens (reviewed in Dubey *et al.*, 2017). Antigens incorporated in immune stimulating complexes (ISCOMs) (Bjorkman *et al.*, 1994, Frossling *et al.*, 2006) were also used for the development of diagnostic ELISAs. In addition, several recombinant antigens predominantly expressed by the tachyzoite stage of potential diagnostic value have been characterised and applied to the ELISA format. Amongst these, recombinant NcSRS2 (Andreotti *et al.*, 2009, Borsuk *et al.*, 2011, Liu *et al.*, 2007), NcSAG1 (Chahan *et al.*, 2003, Howe *et al.*, 2002) and NcGRA7 (Aguado-Martinez *et al.*, 2008, Huang *et al.*, 2007, Jenkins *et al.*, 1997) are the most frequently employed. Almost all currently marketed ELISAs are based on total sonicated tachyzoite lysate antigens; however, tests utilising native NcSRS2 purified protein, native *N. caninum* tachyzoite surface antigen (GP65) captured using monoclonal antibodies or ISCOM-incorporated tachyzoite proteins are also available (Alvarez-Garcia *et al.*, 2013).

Besides enabling the discrimination of infected from uninfected animals, certain ELISA protocols were designed to provide information on the stage of infection and distinguish between acutely and persistently infected animals.

Avidity ELISAs are based on the principle that the first antibodies produced show lower affinity to the antigens than those produced later (Bjorkman *et al.*, 2005). Therefore, they can provide additional information regarding the time elapsed from a primary infection (Bjorkman *et al.*, 2003). Within the investigation of several outbreaks of bovine neosporosis, low avidity IgG responses were correlated to recent primary point-source infections which resulted in epidemic abortions (Basso *et al.*, 2010, Jenkins *et al.*, 2000, McAllister *et al.*, 2000, Schares *et al.*, 2002a). In contrast,

high avidity values are associated with persistent infection (Aguado-Martinez *et al.*, 2005, Bjorkman *et al.*, 2006).

Recombinant antigens expressed by the bradyzoite stage of *N. caninum*, which is found during persistent infections, can also be applied for the determination of the stage of infection.

The combined use of two ELISAs using respectively the bradyzoite stage-specific NcSAG4 and the tachyzoite-expressed NcGRA7 antigen proved useful to discern acute (recent primary infection, recrudescence and reinfection) from persistent infections in cattle (Aguado-Martinez *et al.*, 2008).

None of the ELISA tests, and in general commercial serological tests, use bradyzoite-specific antigens of *N. caninum* (Dubey *et al.*, 2017).

1.7.2.1.2 Indirect Fluorescent Antibody Test (IFAT)

The IFAT has been traditionally considered the reference method for the serodiagnosis of *N. caninum* and has been used for the validation of serological tests based on different principles (Alvarez-Garcia *et al.*, 2003). IFAT protocols are expensive, as they require the *in vitro* production of *N. caninum* tachyzoites, and are laborious (Sinnott *et al.*, 2015). Importantly, the interpretation of the results is subjective and can be affected by the experience and level of training of the operator (Campero *et al.*, 2015).

The IFAT cut-off thresholds differ considerably between laboratories thus hindering the easy comparison of results (Sinnott *et al.*, 2017). Although a 1:200 serum dilution is commonly recommended as cut-off for adult cows, a range of different dilutions has been reported (von Blumroder *et al.*, 2004).

1.7.2.1.3 Neospora Agglutination Test (NAT)

The NAT is based on the agglutination of *N. caninum* whole intact tachyzoites when in the presence of sera containing specific antibodies and is characterised by simple execution (Packham *et al.*, 1998, Romand *et al.*, 1998). However, in a study aimed at comparing commercial and in-house ELISAs, IFATs and a NAT (United States Department of Agriculture, Beltsville, MD, USA, in-house), the latter showed

significantly lower specificity (66.0%) as compared to other tests (Wapenaar *et al.*, 2007c).

1.7.2.1.4 Western blot analysis

WB analysis has been used for the identification of immunodominant tachyzoite antigens by host sera (Alvarez-Garcia *et al.*, 2002) and is generally regarded as a reliable method to detect *N. caninum*-specific antibodies in cattle (Sinnott *et al.*, 2017). Due to the long execution times the technique is not suitable for the screening of large numbers of samples but is useful as a confirmatory method when uncertain results are obtained with different serological tests (Aguado-Martinez *et al.*, 2005). For example, WB analysis is often recommended to retest samples in which borderline levels of fluorescence render the interpretation of IFAT difficult (Ghalmi *et al.*, 2014).

1.7.2.1.5 Immunochromatographic test

A rapid immunochromatographic test using the recombinant NcSAG1 antigen produced in *E. coli* was also developed (Liao *et al.*, 2005) but has not been made commercially available. The future development of lateral flow devices using *N. caninum* antigens for the detection of specific antibodies in whole blood, plasma, serum or milk may represent a possible option to increase the rapidity and applicability to field conditions of the diagnostic tools for bovine neosporosis.

1.7.2.1.6 Recombinant antigens for the serological diagnosis of toxoplasmosis in livestock species and neosporosis in cattle

Serological tests for the diagnosis of toxoplasmosis generally employ a preparation of whole *T. gondii* lysate antigen (TLA) (Holec-Gasior 2014). However, as for the diagnosis of bovine neosporosis, the use of recombinant antigens has been widely investigated.

T. gondii antigens used for the diagnosis of toxoplasmosis in livestock species include recombinant surface antigens (TgSAG1, TgSAG2), dense granule proteins (TgGRA1, TgGRA2, TgGRA6, TgGRA7, TgGRA15), the microneme protein 1 (TgMIC1), the rhoptry proteins 1 (TgROP1) as well as the matrix antigen 1 (TgMAG1) (Ferra *et al.*, 2015). Applied singly or in combination, these recombinant antigens have been used primarily for the development of ELISAs, as well as latex agglutination tests (LATs),

as tools for the detection of *T. gondii* infection in livestock, such as sheep, pigs and horses (Ferra *et al.*, 2015).

Recombinant antigens for the serodiagnosis of *N. caninum* in cattle include NcSAG1 (Chahan *et al.*, 2003, Howe *et al.*, 2002), NcSRS2 (Andreotti *et al.*, 2009, Borsuk *et al.*, 2011, Liu *et al.*, 2007), NcGRA6 (Ghalimi *et al.*, 2014) and NcGRA7 (Aguado-Martinez *et al.*, 2008, Huang *et al.*, 2007, Jenkins *et al.*, 1997). Bradyzoite-specific antigens have also been described, including NcSAG4 (Fernandez-Garcia *et al.*, 2006), NcBRS4 and NcSRS9 (Risco-Castillo *et al.*, 2011).

1.7.2.2 Pre-colostral serology

The detection of *N. caninum*-specific antibodies in the pre-colostral serum of newborn calves can be used to investigate the route of transmission and indirectly assess the infection status of the dam (Dubey *et al.*, 2007, Hietala and Thurmond 1999).

In cattle and other ruminants, transplacental transfer of immunoglobulins does not take place because of the syndesmochorial placenta. Passive immunity is transferred from dam to calf postnatally through the ingestion of colostrum. Therefore, the presence of specific antibody responses in pre-colostral calves is indicative of the activity of the foetal immune system following exposure to the pathogen *in utero* (Innes 2007). Nevertheless, the absence of specific antibodies in stillborn or new-born pre-colostral calves, although suggestive that *N. caninum* infection is unlikely, is not conclusive of absence of infection (Dubey and Schares 2006) since the development of specific antibodies is dependent on the timing of *in utero* infection (Anderson *et al.*, 1997). Effectively, calves infected late during gestation might not have had sufficient time to seroconvert before birth (Dubey *et al.*, 2007).

Given the difficulty of obtaining pre-colostral sera in most field conditions, pre-colostral serology is an impractical diagnostic option. However, it can be very informative for research purposes (Guido *et al.*, 2016).

1.7.2.3 Bulk milk testing

In dairy herds, the detection of *N. caninum*-specific antibodies in bulk milk is useful to provide an estimate of the seroprevalence within the group of animals that contribute to the milk sample (Schaes *et al.*, 2003). Good correlation between bulk milk serological results and the seroprevalence as assessed through testing of individual

serum or plasma samples was reported (Bartels *et al.*, 2005, Milne *et al.*, 2006, Wapenaar *et al.*, 2007a).

The interpretation of bulk milk test results requires specific considerations. Following exposure to a pathogen, milk antibodies appear later and at a lower concentration, approximately 30-fold less, in milk than in serum (Butler 1983). Bulk milk antibody levels are dependent on the proportion of seropositive animals; however, the stage of lactation and the milk yield also influence the concentration of specific antibodies (Frossling *et al.*, 2006).

ELISAs commonly used for testing cattle sera have been adapted for use on bulk milk samples; however, the sensitivity reported is generally limited (Schaes *et al.*, 2004). A minimum of 10-15% serologically positive animals appears to be necessary to produce positive bulk milk testing results (Bartels *et al.*, 2005, Frossling *et al.*, 2006, Wapenaar *et al.*, 2007a). Consequently, the method may underestimate the proportion of infected herds by misclassifying those in which the seroprevalence of *N. caninum* is low as uninfected. Nonetheless, the serological examination of bulk milk is considered a cost-effective and non-invasive diagnostic option which can provide a useful preliminary indication of the *N. caninum* serostatus in dairy herds.

1.7.3 Limitations of current serological diagnostics

Cows infected with *N. caninum* may elude the currently available serological diagnostics due to variations in antibody titres observed throughout the infection, as a result of the dynamics of the host-parasite interaction *in vivo*, in particular in pregnant animals and the intrinsic characteristics of the diagnostic tests used.

This is supported by reports of *N. caninum* serologically negative dams giving birth to seropositive calves or aborting foetuses in which parasite DNA was detected (Mazuz *et al.*, 2014, Sager *et al.*, 2001) as well as post-mortem evidence of *N. caninum* infection found in tissues of seronegative non-aborting cows (Benavides *et al.*, 2012). In cattle, fluctuations in *N. caninum*-specific antibody titres are well documented (Guy *et al.*, 2001, Jenkins *et al.*, 2002, Waldner *et al.*, 1998). These can occur depending on the physiological status of the dam and the activity of the parasite which is dependent on the host immune responses (Innes 2007). In some cases, the antibody levels may

drop below the detection limits of the serological tests so that previously serologically positive animals may become seronegative (Figure 1.3).

A prospective serological study in dairy cattle found that more than one-third of cows, with low antibody titres (1:200) during pregnancy, became serologically negative at the end of gestation (Mazuz *et al.*, 2014). In addition, two studies conducted in Argentina reported that 5% (More *et al.*, 2009, Pare *et al.*, 1996) and 3% (More *et al.*, 2009) of dams that were seronegative at calving gave birth to pre-colostrally seropositive calves.

Most if not all the work reporting fluctuations in specific *N. caninum* antibody concentrations in cattle refers to humoral responses that recognise the tachyzoite stage of the parasite because these are targeted by the vast majority of commercial and in-house diagnostic tests. Concerns about the sensitivity of tests based only on tachyzoite antigens were raised following an interesting observation of a cow whose antibody response was able to recognise bradyzoite-specific but not tachyzoite-specific antigens (Benavides *et al.*, 2012). This animal was kept under experimental conditions as a negative control for a *N. caninum* infection experiment and tested repeatedly negative with commercial and experimental tachyzoite-based ELISAs; however, parasite DNA was detected in several tissues post-mortem, and further serological analysis carried out with a bradyzoite-specific (SAG4) antigen-based ELISA highlighted seropositivity to antigens related to the quiescent bradyzoite stage of *N. caninum*.

In experimentally infected animals, humoral responses to *N. caninum* bradyzoite-specific antigens show individual variability (Aguado-Martinez *et al.*, 2008). Indeed, antibody responses against bradyzoite antigens will depend on the intensity and duration of specific antigen exposure during the host–parasite interaction in cattle. In addition, rupture of tissue cysts may also be necessary to enhance detectable host immune responses against bradyzoite antigens. As suggested for some bradyzoite-specific antigens of the closely related apicomplexan *Toxoplasma gondii*, antigens specific to the quiescent stage may hardly be exposed (Gross *et al.*, 2004). There is no conclusive information on the extent to which these antigens are exposed to the host immune system because this may be difficult to assess and will be subject to individual variability in immunocompetence. The limited immunogenicity observed for the *N. caninum* bradyzoite-expressed BRS4 and SRS9 antigens was ascribed to a possible

late upregulation of expression during persistent infection, with only transient antigenic exposure to the host immune system (Risco-Castillo *et al.*, 2011).

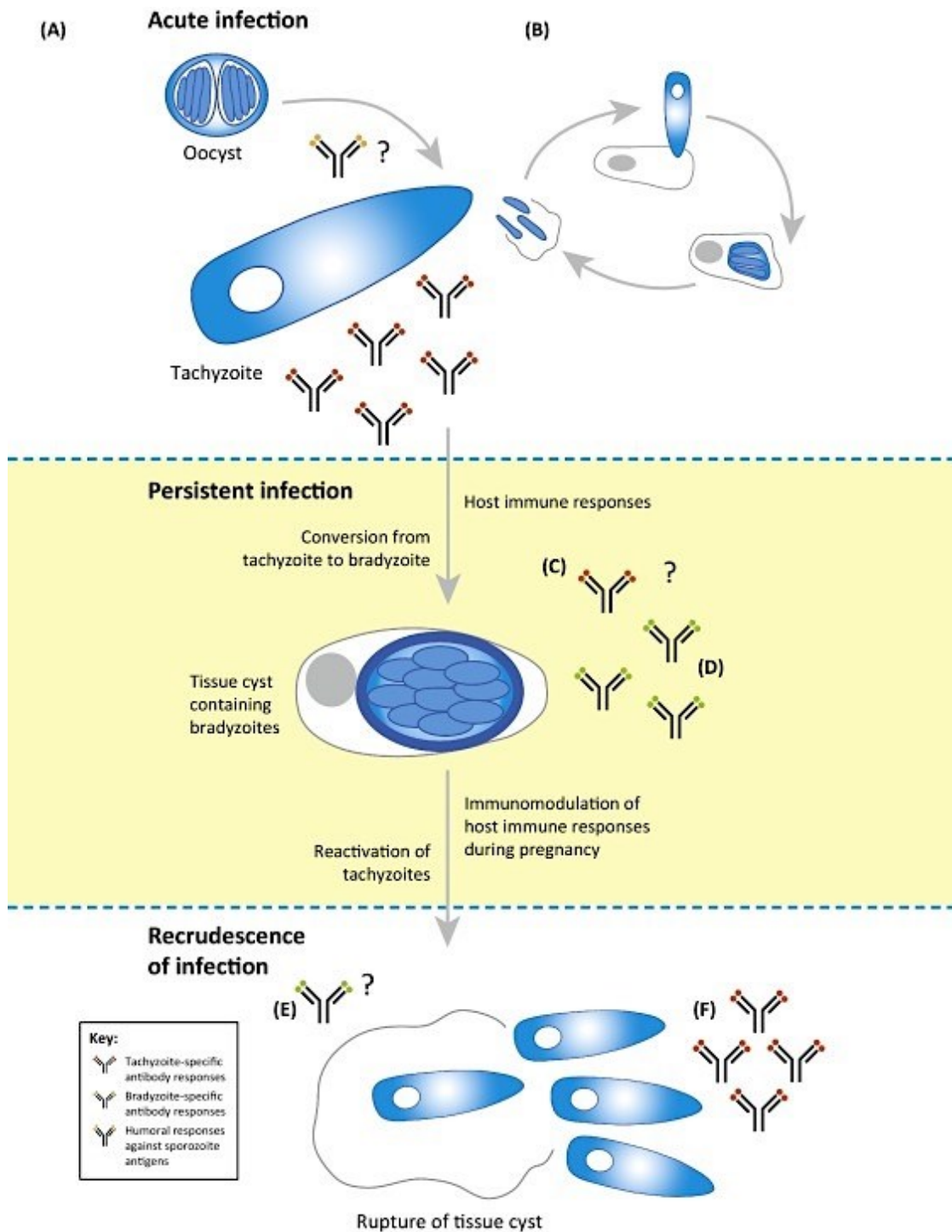


Figure 1.3 – Hypothetical antibody responses following *N. caninum* infection in cattle (adapted from Guido *et al.*, 2016). (A) Ingestion of sporulated oocysts with release of sporozoites may expose the host immune system to sporozoite-specific antigens; there is little information about sporozoite-specific antibody responses. (B) Tachyzoites multiply rapidly by endodyogeny inside several cell types that then rupture triggering the development of tachyzoite-specific antibody responses. These antibody responses are found in most infected animals and are detected by current diagnostic tools that are based on tachyzoite antigens. (C)

Tachyzoites convert into the quiescent bradyzoite/tissue cyst stage, bradyzoite-specific antibodies are produced; however, the quantity and duration of these responses are unknown. (D) During conversion into the bradyzoite/tissue cyst stage, tachyzoite-specific antibody responses may decline below the cut-offs of current diagnostic tests. This may result in false negative results. (E) Immunomodulation during pregnancy may enable reactivation of bradyzoites into tachyzoites that spread throughout the body, thus boosting the tachyzoite-specific immune responses. (F) Antibodies against bradyzoites/tissue cysts may decline at this stage, although there is little information about the dynamics of these stage-specific humoral responses because diagnostic tests targeting bradyzoite-specific humoral responses are not currently used.

In view of the limitations of current diagnostic tests, it has been difficult to assess the frequency and hence the epidemiological impact of animals which are persistently infected with *N. caninum* but test serologically negative. The development of improved diagnostic tests may aid the correct identification of these animals, thus facilitating estimates of the frequency of persistent infection.

1.8 Control of bovine neosporosis

Control programmes for bovine neosporosis have as key objectives the minimisation of the reproductive losses and the reduction of the infection prevalence at the herd level. Current control strategies rely on management techniques aimed at interrupting the life cycle of *N. caninum* at any one or several stages (McAllister 2016). Integrating measures to prevent horizontal transmission from the canid host and methods to reduce the risk of vertical transmission from dam to foetus in infected herds are essential for the implementation of effective control plans (Frossling *et al.*, 2005).

Given the different control options available, an accurate assessment of the impact of the disease at the herd level and a thorough cost-benefit analysis are required before embarking on any disease control efforts (Reichel *et al.*, 2014). The costs of bovine neosporosis encompass the loss of calf revenue from aborted cows, the decreased milk production due to prolonged calving intervals, the costs for rebreeding or eventually replacing aborting cows and the expense associated with investigation of abortion cases. Furthermore, infected herds may be subjected to an overall reduction of the value of the breeding stock (Reichel *et al.*, 2013).

1.8.1 Prevention of horizontal infection

1.8.1.1 General hygiene measures

Measures to minimise the contamination of feedstuff, water and cattle areas with *N. caninum* oocysts are instrumental to prevent postnatal transmission from infected canids. Most epidemiological studies agree that the presence of dogs in breeding cattle premises represents a risk factor for bovine neosporosis; therefore, the number of dogs on farm should be limited and the access of stray canids should be controlled. In infected herds, adult farm dogs are likely to have already experienced *N. caninum* infection and may be less likely to shed oocysts (Antony and Williamson 2003). However, dog puppies, which are likely to be naïve to the parasite, are considered to have a higher risk of producing oocysts following infection. No dogs, but especially litters of puppies, should have access to cattle and feedstuff storage areas; dog proof fencing and the protection of feed storage areas and feed mixing equipment from dog faecal contamination are highly recommended. Likewise, the protection of sources of

drinking water using elevated troughs with potable water may help in preventing cattle exposure to *N. caninum* oocysts (McAllister 2016).

Since dogs can acquire the infection by ingesting infected bovine tissues, the prompt removal and appropriate disposal of aborted fetuses, afterbirths as well as dead livestock is important to stop the parasite life cycle (Basso *et al.*, 2001, Dijkstra *et al.*, 2001a). Furthermore, dogs should not be fed raw ruminant tissues as dog food as it may contain *N. caninum* tissue cysts (Cavalcante *et al.*, 2011).

The detection of *N. caninum* DNA in tissues from feral mice and rats suggested that these species can act as intermediate hosts for the parasite. Although there is no definitive evidence, rodents may represent a source of infection to domestic and wild canids and play a role in maintaining and spreading neosporosis (Ferroglia *et al.*, 2007, Jenkins *et al.*, 2007). Therefore, on farm rodent control would be also recommended. This would also reduce the risk of leptospirosis thus producing additional benefits within the control of reproductive losses in cattle in general (Reichel *et al.*, 2013). Similarly, a potential role of free-range poultry in the epidemiology of *N. caninum* has been suggested. The presence of chickens, which can be infected with the parasite (Costa *et al.*, 2008), was correlated to an increased risk of bovine abortion (Bartels *et al.*, 1999) and increased seroprevalence in cattle (Otranto *et al.*, 2003).

1.8.2 Management of vertical transmission

Serological testing associated with the adequate management of positive animals is the most common method to limit endogenous transmission within a herd (Guido *et al.*, 2016). Infected cows can be removed from the herd (test-and-cull regime) or excluded from breeding to generate replacement stock (selective breeding) (Reichel *et al.*, 2014).

The reliable identification of all *N. caninum*-infected animals requires serological tests in which the cut-off thresholds are adjusted to provide the maximum level of diagnostic sensitivity (Reichel and Ellis 2002). However, the possibility of false negative results which may affect the effectiveness of the disease control measures implemented should be considered.

1.8.2.1 Test and cull

Culling *N. caninum*-seropositive dams was shown to reduce the within-herd prevalence over time in farms in which adequate measures to prevent horizontal infection were in place (Frossling *et al.*, 2005, Hall *et al.*, 2005).

Some authors questioned whether retaining infected animals would be more beneficial than aiming at a herd in which all animals are completely naïve to the pathogen hence more susceptible to horizontal infections (Innes *et al.*, 2000, McAllister *et al.*, 2000). This concern arose from the observation that cattle can develop partial immunity to the parasite and the risk of abortion tends to decrease over time in cows repeatedly exposed to the parasite (Innes *et al.*, 2000). Although repeated environmental exposure to *N. caninum* can eventually lead to a lower risk of abortion, there is evidence that such risk is still higher than in seronegative animals (Reichel *et al.*, 2013). Most importantly, these dams are still able to transmit the parasite to their offspring thus maintaining the infection in the herd and deferring the abortion risk to the next generation (Reichel *et al.*, 2013).

Due to the high costs involved, culling seropositive animals may be economically sustainable only in herds with low seroprevalence in which only a small proportion of animals would need to be removed.

1.8.2.2 Selective breeding

The exclusion of heifers born from antibody-positive dams from breeding reduces the risk of *in utero* infections at the herd level (Frossling *et al.*, 2005). This control option is effective provided that endogenous vertical transmission is the predominant route of infection and post-natal exogenous transmission is negligible (Hall *et al.*, 2005, Landmann *et al.*, 2011).

In farms with high *N. caninum* seroprevalence ($\geq 50\%$), avoiding breeding replacement stock from seropositive dams was shown to be economically sustainable (Hasler *et al.*, 2008). Within a cost-benefit simulation model investigating different control options in endemically infected beef herds, the best economic return was provided by the exclusion of the daughters of seropositive dams as potential replacements (Larson *et al.*, 2004).

Serological analysis of all potential replacement heifers, regardless of the serological status of their dams, could be performed to increase the level of confidence that the prospective breeding females do not harbour *N. caninum*. Serology can be done pre-colostrum ingestion, but is most commonly carried out after 6 months of age when maternal antibody levels, from passive transfer via colostrum, have declined (McAllister 2016).

1.8.2.3 Testing replacement breeding stock

Purchased cows and heifers represent a risk of introducing *N. caninum* into the herd. As a rule, replacement breeding stock should be obtained from herds with outstanding breeding performances and no history of bovine neosporosis (Roelandt *et al.*, 2015). Serological testing of prospectively purchased animals should be performed. Due to the possibility of false negative results, some authors recommend repeat testing 4-6 weeks after introduction to the herd and re-testing doubtful samples using WB analysis (Alvarez-Garcia *et al.*, 2003, Ortega-Mora *et al.*, 2006). Antibody testing the mothers of the animals whose daughters are to be introduced into the herd may increase the chances of identifying individuals which test falsely negative and infected family lines in which the parasite is vertically transmitted.

1.8.2.4 Reproductive management

Embryo transfer (ET) from seropositive donors to seronegative recipients can prevent endogenous transmission of *N. caninum* (Campero *et al.*, 2003, Landmann *et al.*, 2002) as during the preimplantation stages, the zona pellucida protects the embryos from the parasite (Bielanski *et al.*, 2002). Furthermore, there are several reports of successful recovery and implantation of uninfected embryos taken from infected dams which stopped the cycle of the parasite (Baillargeon *et al.*, 2001, Campero *et al.*, 2003, Landmann *et al.*, 2002). The limiting factors of the use of ET are the high costs which may be justified only by the value of the future calf in high genetic merit cattle herds. A significant reduction in the abortion risk was observed in *N. caninum* seropositive high-producing dairy cattle inseminated with beef bull semen (Lopez-Gatius *et al.*, 2005). This was attributed to the favourable effect of cross-breeding on foetal health and placental function (Almeria *et al.*, 2009, Lopez-Gatius *et al.*, 2005). Although ineffective to prevent transmission *in utero*, this management strategy ensures that

breeding replacements from infected cattle are removed because female dairy × beef crosses are not usually retained for milk production.

1.8.2.5 Reduction of predisposing factors

Given the role of the host immune responses in the control and reactivation of *N. caninum*, concomitant infections, management factors and other stressors, which may cause immunosuppression, may be implicated in the reactivation of bradyzoites to tachyzoites with subsequent vertical transmission. Some authors suggested that infectious diseases, such as bovine rhinotracheitis (IBR) and bovine viral diarrhoea (BVD), characterised by immunosuppression may lead to recrudescence of *N. caninum* and foetal infection in pregnant dams (Reichel *et al.*, 2013). Although the absence of BVD was shown to have a protective effect against *N. caninum* infection (Vanleeuwen *et al.*, 2010), in several studies an increased risk of abortion in cows seropositive for both pathogens was not observed (Bjorkman *et al.*, 2000, Mainar-Jaime *et al.*, 2001, Stahl *et al.*, 2006). Nevertheless, the control of concomitant reproductive infections may help reducing the impact of neosporosis at the herd level. The immunosuppressive effects of mycotoxins may also have a role in the recrudescence of a persistent *N. caninum* infection (Bartels *et al.*, 1999, Dubey *et al.*, 2007). Consequently, providing pregnant dams with good quality and properly stored feed and fodder is recommended (McAllister 2016).

1.8.3 Other approaches

1.8.3.1 The “live with the disease” option

In herds in which the prevalence of neosporosis is low, the costs related to specific control measures may exceed the economic losses incurred by the disease. For this reason, a non-interventionist “live with the disease” policy may be the preferable course of action in some farms (Reichel *et al.*, 2013). In one study based on mathematical modelling, Reichel and Ellis (2006) indicated a within-herd prevalence of 18-21% as the threshold above which the benefits of test and cull programmes would significantly outweigh the costs involved in terms of serological testing and buying replacements.

1.8.3.2 Vaccination

Economic analysis suggested that vaccination might be the most efficient intervention strategy to control neosporosis in cattle (Reichel and Ellis 2006); however, registered vaccines are currently not available. Several years ago, a vaccine based on inactivated *N. caninum* tachyzoites, Bovilis Neoguard® (Intervet International B.V., Boxmeer, The Netherlands), was registered in several countries but subsequently withdrawn from the market due to the low efficacy (Weston *et al.*, 2012).

The development of an effective vaccine is certainly one of the primary goals within the bovine neosporosis research and considerable resources are invested in the investigation of vaccine candidates using both mouse and ruminant models (reviewed in Hemphill *et al.*, 2016, Horcajo *et al.*, 2016).

Live-attenuated vaccines showed promising results in different studies. Intravenous and subcutaneous inoculation of cattle with tachyzoites of the attenuated Nc-Nowra strain protected pregnant cows against foetopathy by inducing strong antibody and cellular responses with the production of IFN- γ (Weber *et al.*, 2013, Williams *et al.*, 2007). The naturally attenuated Nc-Spain1 isolate, inoculated prior to artificial insemination, produced protection against foetal death in 50% of the animals subsequently challenged with the Nc-1 strain in early (day 70) gestation. In addition, significantly lower pre-colostral antibodies were observed in asymptomatic calves born from dams challenged during mid-gestation (day 135) (Rojo-Montejo *et al.*, 2013). Another study reported a significantly lower incidence of abortion in cows vaccinated at mid-gestation with a live isolate denominated Ncls491 (16%) compared to the unvaccinated group (26%). In the same trial, no differences in the seroprevalence in pre-colostral calves were observed between the vaccinated and unvaccinated groups (Mazuz *et al.*, 2015).

Subunit vaccines have also been developed; however, limited efficacy was observed. Non-pregnant cattle immunised with recombinant NcGRA7 combined with oligo-mannose microsomes (M3-NcGRA7) showed enhance humoral and cell-mediated immune responses as well as a reduced parasite burden in the brain following challenge with *N. caninum* tachyzoite (Nc-1 strain) (Nishimura *et al.*, 2013b). In a study carried out in pregnant cattle challenged at day 70 of pregnancy, immunisation with recombinant NcSAG1, NcHSP20 and rNcGRA7, incorporated in ISCOMs

elicited a marked humoral immune response but failed to prevent vertical transmission (Hecker *et al.*, 2014).

In future years, genomic and transcriptomic resources would play a primary role in the *in silico* identification of a priority list of potential vaccine candidates which could be experimentally assessed (Goodswen *et al.*, 2013). It is likely that any killed or sub unit vaccine will require an adjuvant or delivery system that will enable a long-lasting cell-mediated immune response to be induced.

1.8.3.3 Antiprotozoal treatment

Despite the assessment of the efficacy of several drugs against *N. caninum* both *in vitro* and *in vivo* in laboratory animals, there are no antiprotozoal compounds licensed for use in cattle to treat neosporosis (Muller and Hemphill 2013).

Toltrazuril is effective against various coccidians and extensively used for the treatment of coccidiosis in poultry and cattle; however, limited effectiveness was observed against *N. caninum in vivo* (Haerdi *et al.*, 2006).

Artemisinin and derivatives, dicationic pentamidine and spiroindolones are the most promising drug candidates (Hemphill *et al.*, 2016).

Nonetheless, it appears unlikely that the pharmacological treatment of *N. caninum* infection in cattle would be cost-effective. Given the pathogenesis of bovine neosporosis, preventing abortions would imply long-term administrations resulting in an increased risk of unacceptable residues in milk or meat. Furthermore, the establishment of withdrawal periods would represent an additional production cost (Dubey *et al.*, 2007, Dubey *et al.*, 2017).

1.9 Aims of the thesis

Prophylaxis through vaccination is generally considered the desirable approach for the control of bovine neosporosis (Monney and Hemphill 2014). However, effective vaccines may not be commercially available for several years to come (Reichel *et al.*, 2014). Furthermore, despite the promising effects against *N. caninum* displayed by several antiprotozoal compounds both *in vitro* and *in vivo* (Mazuz *et al.*, 2012, Muller *et al.*, 2015, Muller and Hemphill 2011, Schorer *et al.*, 2012, Winzer *et al.*, 2015), the chemotherapeutic treatment of neosporosis in cattle is not a popular option because of the prospective high costs and the risk of unacceptable residues in meat and milk (Dubey *et al.*, 2007).

Consequently, the control options aimed at reducing the impact of bovine neosporosis are restricted to management practices in which the key role is played by the reliable discrimination of infected from uninfected animals (Reichel *et al.*, 2013). For this purpose, serological diagnosis is widely recognised as the approach of choice. Additionally, the investigation of the genetic diversity of *N. caninum* can provide valuable information on the source of infection and the predominant route of transmission at the herd level, thus helping to shape effective control strategies (Basso *et al.*, 2010).

The capacity of *N. caninum* to establish persistent infections sustained by the quiescent bradyzoite stage represents one of main challenges to the accurate identification of all infected animals. In fact, the level of antibodies against the tachyzoite stage, which proliferate actively during acute infections, may decline below the detection limits of current serological tests when the infection becomes quiescent or at certain stages in pregnant animals.

Most, if not all, commercially available tests are based exclusively on tachyzoite antigens (Alvarez-Garcia *et al.*, 2013). These tests may not always identify cattle in which tachyzoite antibody responses have declined but a test where both tachyzoite and bradyzoite antigens were used may help to improve the consistent detection of all animals harbouring the parasite.

In order to improve the current diagnostic options to reliably detect *N. caninum* infection in cattle and to help the development of strategies to control the disease, the research in this thesis has the following objectives:

1. Identify proteins expressed by the bradyzoite stage of *N. caninum* which could be employed for the development of serological assays to detect cattle persistently infected with *N. caninum* (Chapter 2)
2. Evaluate different recombinant *N. caninum* stage-specifically expressed antigens for the detection of specific antibodies in cattle (Chapter 3)
3. Determine the seroprevalence of bovine neosporosis in British dairy cattle (Chapter 3)
4. Develop a molecular typing tool which could be applied for the study of the genetic diversity of *N. caninum* within the investigation of abortion outbreaks caused by the parasite (Chapter 4)
5. Explore perceptions and approaches to the diagnosis and control of bovine neosporosis in current veterinary practice in the United Kingdom (Chapter 5).

Chapter 2: Identification and expression of *N. caninum* bradyzoite-expressed antigens for ELISA development

2.1 Introduction

A key to the success of *N. caninum* as a parasite is its ability to establish life-long persistent infections in which the bradyzoite stage survives, relatively quiescent, enclosed in tissue cysts (Hemphill *et al.*, 2006). During pregnancy, persistent infections can recrudesce with re-conversion of bradyzoites into the actively multiplying tachyzoites that initiate an acute infection similar to that occurring after a primary exposure to the parasite. This may lead to invasion of the gravid uterus and vertical transmission to the foetus that can result in abortion or the birth of congenitally infected offspring (Williams *et al.*, 2009). Although asymptomatic, persistently infected cattle are able to transmit the parasite to their progeny over consecutive pregnancies, perpetuating the infection within a herd over generations (Innes 2007). In particular, where congenitally infected but clinically healthy heifers are retained for breeding, spreading of the parasite vertically in the herd can take place very efficiently (Bjorkman *et al.*, 1996).

Given the current lack of effective vaccines and licensed antiprotozoal treatments (Goodswen *et al.*, 2013), the control of bovine neosporosis relies exclusively on biosecurity and management practice whose effectiveness depends on the correct classification of infected and uninfected animals. Alongside the identification of acutely infected cattle, the detection and appropriate management of persistently infected animals would help significantly in the control of the disease. In addition, the discrimination between acutely and persistently infected cattle provides epidemiological information that allows identifying the predominant transmission pattern (horizontal or vertical) thus the major source of infection within a herd. Such information is useful for the selection of optimal control strategies (Aguado-Martinez *et al.*, 2008).

Serological diagnostic tests, primarily ELISAs and IFAT, which are used routinely for the diagnosis of *N. caninum* infection in live animals (reviewed in Dubey and Schares

2006) are based on a variety of tachyzoite antigen preparations, both native and recombinant. Generally considered adequate, currently available serological diagnostics may fail to detect those animals in which specific antibody titres against the tachyzoite stage may have dropped below the cut off limits (reviewed by Dubey and Schares 2006). Antibody levels against *N. caninum* tachyzoite antigens fluctuate depending on the stage of pregnancy and the age of the animal (Conrad *et al.*, 1993b, Waldner *et al.*, 1998). During persistent infections, in which *N. caninum* has undergone stage conversion into bradyzoites, the host immune system is no longer exposed to the tachyzoites and specific antibody responses may decline to a level they are no longer detectable (Guido *et al.*, 2016).

Diagnostic tools that measure the avidity of *N. caninum*-specific antibodies have been proposed for the discrimination between acute and persistent infection (Bjorkman *et al.*, 1999, Sager *et al.*, 2003, Schares *et al.*, 2002a). These tests are based on the principle that the affinity of specific immunoglobulins for the antigens varies depending on the time elapsed after infection. Low avidity is associated with recent infection whereas high avidity is indicative of persistent infection (Aguado-Martinez *et al.*, 2005, Bjorkman *et al.*, 1999). Nevertheless, the rapid increase of antibody avidity after a primary infection hampers the discrimination between persistent infection and recrudescence (Bjorkman *et al.*, 2006, Schares *et al.*, 2002a). In addition, all reported avidity tests are based exclusively on tachyzoite antigens and may fail in identifying those animals in which antibody responses against the tachyzoite may have declined, similarly to currently available non-avidity tests.

Based on differences in gene transcription, different protein expression patterns were reported between *N. caninum* tachyzoites and bradyzoites (Kang *et al.*, 2008a, Kang *et al.*, 2008b). As a result, the two infectious stages of the parasite display a different antigenic repertoire. This variation in the antigenic composition is also known in *T. gondii*, which is very closely related to *N. caninum*, in which the expression of numerous genes in a stage-specific manner is well documented (Lyons *et al.*, 2002). For example, genes of the surface antigen (SAG) 1-related sequence (SRS) superfamily, that encode GPI-anchored surface proteins, are differentially expressed in tachyzoites and bradyzoites as mostly non-overlapping sets (Jung *et al.*, 2004). A number of stage-specific *T. gondii* antigens have been described. Surface antigens,

including TgSAG1, TgSAG2A, TgSAG2B, TgSRS1 and TgSRS3 are present only on the tachyzoites whereas TgBRS4 (Van *et al.*, 2007) TgSRS9 (Kim and Boothroyd 2005) TgSAG4 and TgSAG4.2 (Odberg-Ferragut *et al.*, 1996) are expressed exclusively by the bradyzoites. An immunogenic heat shock protein named TgBAG1 was also found to be specifically expressed by the bradyzoite stage (Bohne *et al.*, 1995). In addition, structural antigens of the tissue cyst wall such as CST1 (Zhang *et al.*, 2001) and the tissue cyst matrix (TgMAG1) (Ferguson and Parmley 2002, Parmley *et al.*, 1994) were also described.

Antigens that are expressed by the bradyzoite stage can be used for the detection of specific antibody responses in those persistently infected animals that harbour the parasite at the quiescent stage. Specific antibody responses to the TgBAG1 and TgMAG1 antigens were detected in murine and human infection models (Mun *et al.*, 1999, Parmley *et al.*, 2002).

In *N. caninum*, several bradyzoite-specific proteins have been identified and characterised to date. NcSAG4 was the first described bradyzoite-specific antigen (Fernandez-Garcia *et al.*, 2006) that was used for the serodiagnosis of *N. caninum* infected cattle (Aguado-Martinez *et al.*, 2008, Hu *et al.*, 2011). Other antigens including NcBSR4 (Risco-Castillo *et al.*, 2007), NcMAG1 (Guionaud *et al.*, 2010), NcSRS9 (Risco-Castillo *et al.*, 2011) and NcBAG1 (Kobayashi *et al.*, 2013) were subsequently reported.

The parallel use of a NcSAG4-based ELISA and a similar ELISA based on the NcGRA7 antigen, considered to be a marker of acute infection, has been shown to be useful for discriminating chronic/persistent infection from acute primo-infection or recrudescence (Aguado-Martinez *et al.*, 2008). Nevertheless, to date, there are no commercially available diagnostic tests based on *N. caninum* bradyzoite antigens.

For these reasons, expanding the array of stage-specific antigens that could be used for the development of novel diagnostic tools may help to increase the sensitivity of the serological diagnosis of bovine neosporosis with the aim of improving the control of the disease (Huang *et al.*, 2007).

The aims of this study were to:

1. Identify target genes putatively expressing *N. caninum* bradyzoite antigens
2. Express candidate genes as recombinant proteins using bacterial expression systems
3. Assess the immunoreactivity of recombinant antigens
4. Develop diagnostic ELISAs to detect specific antibodies produced against the bradyzoite stage of *N. caninum* using bradyzoite-expressed recombinant antigens.

2.2 Materials and methods

2.2.1 Identification of *N. caninum* candidate genes

A panel of *N. caninum* genes, putatively encoding antigens that are expressed by the bradyzoite stage, was selected using the closely related apicomplexan *T. gondii* as a model. Most of the knowledge on the antigenic repertoire of *N. caninum* was derived from research on the more extensively studied parasite *T. gondii* (Howe and Sibley 1999). A great wealth of information, in particular regarding the *in vitro* stage-related gene expression profiles, is currently available for *T. gondii* (Lyons *et al.*, 2002). In addition, the availability of the completely sequenced and annotated *T. gondii* (GT1, ME49 and VEG strains) (Kissinger *et al.*, 2003) and *N. caninum* (Liverpool strain) genomes (Reid *et al.*, 2012) offers an invaluable resource for the study of both parasites.

The identification of candidate genes was carried out using two different approaches: (1) gene orthology and (2) protein homology.

T. gondii and *N. caninum* genomes show a high degree of synteny (i.e. the physical co-localisation of genetic *loci* on the same chromosome amid related species) with a one-to-one correspondence between most protein-coding genes. Although divergences have been observed, many *T. gondii* proteins have orthologous proteins encoded within the genome of *N. caninum* (Reid *et al.*, 2012).

Using the *T. gondii* genome, maintained by the Eukaryotic Pathogen Database Resource Centre and publicly available in ToxoDB (<http://www.toxodb.org>) (Gajria *et al.*, 2008), *T. gondii* protein coding genes were selected based on their expression at the bradyzoite stage as evidenced by the bradyzoite *in vitro* and/or *in vivo* transcriptome (ME49 strain). The corresponding orthologues were then identified within the *N. caninum* genome which is also available in ToxoDB (Table 2.1); the database was consulted in January 2014.

The focus was on protein coding genes that putatively belonged to the SRS (SAG1-related sequences) family since this includes the major surface antigens in both *T. gondii* and *N. caninum*.

Where corresponding orthologues could not be located, homology searches of *T. gondii* antigens known to be expressed by the bradyzoite stage or to constitute the

tissue cyst or the tissue cyst wall were performed. The amino acid sequences of known and already characterised bradyzoite expressed *T. gondii* antigens were searched against the *N. caninum* database for the upstream identification of genes encoding homologous proteins (Table 2.2). Protein homologues of TgSRS44 and TgSRS13 were selected.

TgSRS44, also known as CST1, is one of the major components of the *T. gondii* tissue cyst wall that is induced during the bradyzoite development and is believed to promote bradyzoite persistence by conferring mechanical resistance to the tissue cyst wall (Tomita *et al.*, 2013). High similarity between the TGME49_264660 *T. gondii* gene encoding SRS44 also known as CST1 and the NCLIV_040495 *N. caninum* gene were observed. The overall identity between the two amino acid sequences was 74% (query cover 82%).

TgSRS13, is a bradyzoite-specific antigen that has been already characterised in *T. gondii* (Reid *et al.*, 2012); therefore, its homologue was considered a good candidate for the prospective development of serological tests. At the amino acid level, the homology between TGME49_222370 encoding TgSRS13 and the *N. caninum* homologue NCLIV_005760 was 56% (query cover 62%).

Table 2.1 – *N. caninum* genes selected based on gene orthology with *T. gondii* bradyzoite-expressed antigens.

<i>Toxoplasma gondii</i> Gene tag	Product description	<i>Neospora caninum</i> Orthologue Gene tag
TGME49_321490	SAG-related sequence (SRS12D)	NCLIV_004430
TGME49_267130	SAG-related sequence (SRS38A)	NCLIV_038900
TGME49_308840	SAG-related sequence (SRS51)	NCLIV_053310
TGME49_280570	SAG-related sequence (SRS35A)	NCLIV_019580
TGME49_281930	SAG-related sequence (SRS39)	NCLIV_023620

Table 2.2 – *N. caninum* genes selected based on protein homology with *T. gondii* known antigens that are expressed by the bradyzoite/tissue cyst stage.

Reference	<i>Toxoplasma gondii</i>		<i>Neospora caninum</i>	
	Gene tag	Product description	Homologue protein Gene tag	Product description
Tomita <i>et al.</i> , 2013	TGME49_264660	SAG-related sequence (SRS44)	NCLIV_040495	SRS-domain containing protein
Reid <i>et al.</i> , 2012	TGME49_222370	SAG-related sequence (SRS13)	NCLIV_005760	putative SRS13

2.2.2 Selection of regions of interest within candidate genes

Independently of the approach taken for identification, all predicted amino acid sequences encoded by the candidate genes were screened for B cell epitopes in order to increase the chances of selecting immunogenic proteins recognised by antibodies. The presence and location of linear (i.e. single continuous stretch of amino acids within a protein sequence) B cell epitopes was initially predicted using the BepiPred method (<http://www.cbs.dtu.dk/services/BepiPred>) (Larsen *et al.*, 2006). Since most B cell epitopes are conformational (i.e. discontinuous set of amino acids brought into physical proximity by protein folding) (Barlow *et al.*, 1986) the amino acid sequences were also screened for conformational B cell epitopes using the CBTOPE prediction tool (<http://www.imtech.res.in/raghava/cbtope>) (Ansari and Raghava, 2010). Regions within candidate genes containing at least one predicted linear or conformational B cell epitope were selected (Figure 2.1).

For each gene identified, the relative hydrophobicity/hydrophilicity of the correspondent amino acid sequence was evaluated using the ProtScale tool (<http://www.web.expasy.org/protscale>) based on the Kyte-Doolittle method (Kyte and Doolittle 1982) (Figures 2.2-2.3). In most cases, N-terminal signal peptides, C-termini and highly hydrophobic regions at the extremities of the protein of interest were excluded in order to increase the likelihood of obtaining a water-soluble final product. Soluble proteins are desirable as they are more easily purified when produced in bacterial expression systems.

Nucleotide sequences encoding the truncated predicted proteins of interest were then checked to ensure that they were included within a single exon. Where introns were

present, shorter or alternative fragments were selected. Furthermore, the truncated amino acid sequences were checked for gaps, resulting from the presence of stop codons within the corresponding nucleotide sequence, using the EMBOSS Sixpack DNA sequence translation software (http://www.ebi.ac.uk/Tools/st/emboss_sixpack).

> NCLIV_004430

```

1 .....010.....020.....030.....040.....050.....060
2 MARTGRTQHR  RRGFRSTARK  LMAVCLDGV  LFSNGGAVAG  DPVEGLLRRE  LSPDSSDAQG
3 #####
4 4443344433  3433444443  3333233333  2333333333  3333443443  3333333223

1 .....070.....080.....090.....100.....110.....120
2 ESGIAGVVAT  CTVASSGDAV  TRPATTLTLS  SSTLVATLEC  MGQSMKALPA  DAATVCVAEA
3 #####
4 3333333333  3333333333  3344333333  3333333332  2443443433  3333333333

1 .....130.....140.....150.....160.....170.....180
2 TASKELNSSN  CSIGDTSMGN  PVKLQELLGT  NDPVQWK AIS  SSDGKNQGES  RMLELSEASL
3 #####
4 3333333344  3344444443  3344333333  4545555555  4443333333  3333223332

1 .....190.....200.....210.....220.....230.....240
2 PRTDKKFMVG  CQSGEAGENK  SCQVTNVNNA  KPSSVDDKNA  VTCAYGKDSN  PEAVEVEMSE
3 ###
4 2444454444  4555544444  4554443333  3333334433  4455565444  4433333334

1 .....250.....260.....270.....280.....290.....300
2 DKNTLTIDCG  KGGSMPAEF  TSQYCSPEGD  TFEDCSKANY  SDVLPFAST  WWTNTENKTQ
3 #####
4 4444444433  2234445444  4443444446  6655565433  3444444443  3333333333

1 .....310.....320.....330.....340.....350.....360
2 ATLTIPTKDF  PPEDQRLLLG  CVPKTQNADQ  STREENLTES  VSETSSCRVL  VTVKAASSAS
3 #####
4 3333333333  3323333333  4543355544  3333333333  3333433333  3433333343

1 .....370.....380.....
2 AVSFTPQAVA  ATSGAVLLAG  LFSGSL
3 #####
4 3333333333  3333433332  333333

```

Figure 2.1 – Example of selection of target regions based on predicted linear and conformational B cell epitopes within NCLIV_004430 (NCLIV_004430-A corresponded to the whole sequence, regions NCLIV_004430-B and NCLIV_004430-C are highlighted in yellow and blue respectively, overlapping region in green).

Line 1 – amino acid position, line 2 – amino acid sequence, line 3 – linear B cell epitopes prediction: (#) likely epitope residues (.) unlikely epitope residues (<http://www.cbs.dtu.dk/services/BepiPred>), line 4 – conformational epitopes prediction (probability scale 0-9, if > 4 the amino acids can be considered as epitope residues, underlined in red) (<http://www.imtech.res.in/raghava/cbtope>).

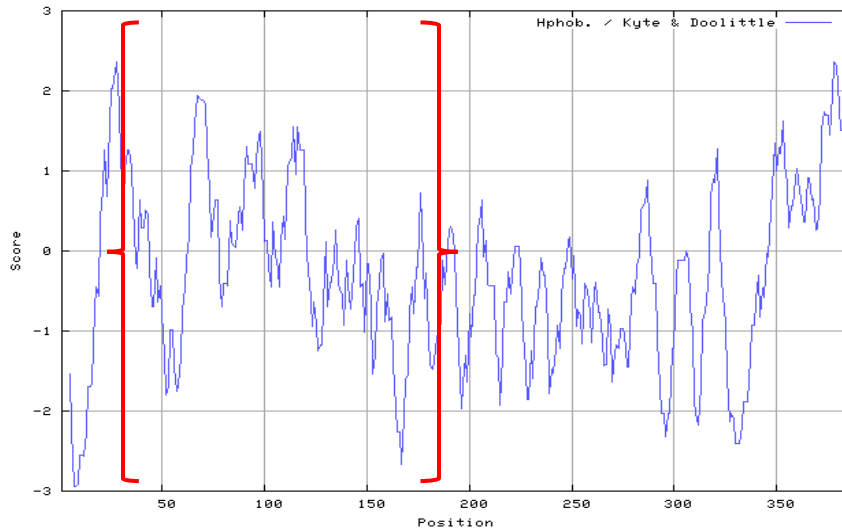


Figure 2.2 – Hydrophobicity plot of the amino acid sequence of NCLIV_004430, in red parentheses the region NCLIV_004430-B selected and expressed as a recombinant protein (tNcSRS12A-B).

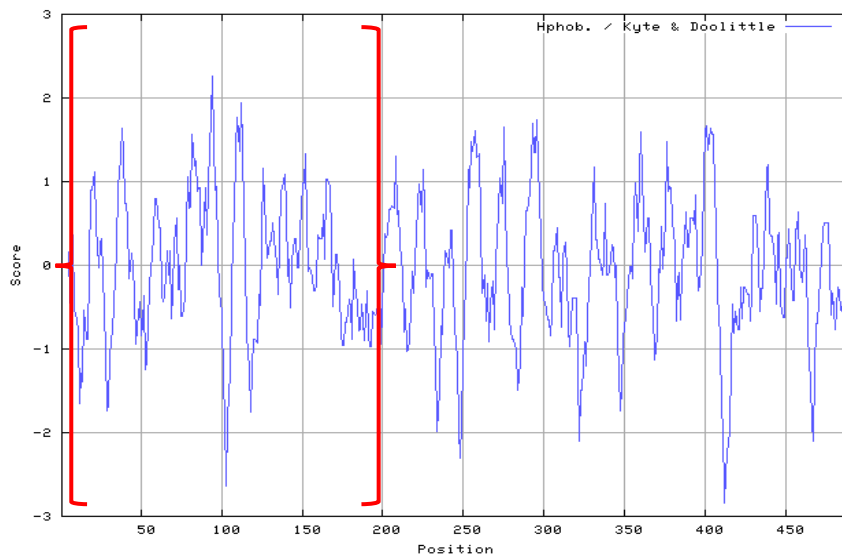


Figure 2.3 – Hydrophobicity plot of the amino acid sequence of NCLIV_040495 (first 500 amino acids), in red parentheses the region NCLIV_040495-A selected and expressed as a recombinant protein (tNcSRS44-A).

2.2.3 PCR amplification

Selected sequences were amplified by PCR from *N. caninum* (NC-1 strain) DNA. *N. caninum* tachyzoites were cultured on confluent Vero cell monolayers grown for 24 h in RPMI 1640 (Sigma-Aldrich, Munich, Germany) supplemented with 2 mM glutamine (Thermo Fisher Scientific, Waltham, MA, USA), 2% foetal bovine serum (FBS), 100 IU/ml penicillin and 50 IU/ml streptomycin (Sigma-Aldrich, Munich, Germany), as previously described (Innes *et al.*, 1995). Cell monolayers were inoculated with an adjusted multiplicity of infection (MOI) of *N. caninum* tachyzoites. Tachyzoites were maintained by serial passage onto new Vero cells monolayers every 3-4 days, harvested by disruption with a sterile cell scraper (Corning, USA) and washed 3 times by resuspension and centrifugation at 706×g for 5 minutes at 4°C. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer's instructions.

Intra-sequence specific PCR primers were designed for each selected target region. Restriction enzyme cleavage sites analysis was carried out (<http://tools.neb.com/NEBcutter2>) and cross checked with the restriction sites available in the pQE-30, pQE-31 or pQE-32 vector systems (Qiagen, Manchester, UK). The choice between the pQE-30, pQE-31 or pQE-32 depended on whether the open reading frame (ORF) was conserved upon insertion of the target sequences. Suitable restriction sites were added at the 5' end of the primers. Designed primers (Table 2.3) were manufactured by Eurofins MWG Operon and purified using the high purity salt free (HPSF) method by the manufacturer (Eurofins Genomics, Ebersberg, Germany).

Each PCR reaction was performed with 2.5 µl genomic DNA (0.25 µg) or 2.5µl DNase/RNase free water (negative control), 5 µl 10× custom PCR buffer – SM0005 (45 mM Tris-HCl, 11 mM (NH₄)₂SO₄, 4.5 mM MgCl₂, 0.113 mg/ml bovine serum albumin (BSA), 4.4 µM EDTA and 1.0 mM dATP, dATC, dGTC, dTTP) (ABgene, Epsom, Surrey, UK), 0.75 units BioTaq (Bioline, London, UK) and 0.05 µM of forward and reverse primers. The reaction was made to a final volume of 50 µl with DNase/RNase free water.

Initial denaturation (94°C for 5 min) was followed by 35 cycles of denaturation (94°C for 45 s), annealing (from 56 to 59°C, depending on the set of primers, for 45 s) and elongation (72°C for 45 s) with a final extension at 72°C for 5 min. Optimum annealing temperatures were determined by gradient PCR reactions for each set of primers (Table 2.3).

The PCR products were electrophoresed on a 1.8% (w/v) agarose/TAE gel (Appendix I) incorporating Gel Red™ (Biotum, Hayward, CA, USA) and visualised under UV light. A 1kb DNA ladder (Promega, Madison, WI, USA) was used to identify relevant amplicons that were excised from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's specifications. Purified PCR products were stored at -20°C until cloning.

Gene ID-region	Primers	Restriction site added	Ta (°C)	Amplicon size (bp)
NCLIV_004430-A	F 5'- <i>gcatgc</i> ATGGCGAGAACGGGCAGAACG-3' R 5'- <i>aagctt</i> GAGGGAACCCGAGAAGACCCC-3'	<i>Sph</i> I <i>Hind</i> III	57	1,170
NCLIV_004430-B	F 5'- <i>gcatgc</i> GCAGTTTGTGGACGGAG-3' R 5'- <i>aagctt</i> GCTTTGACAGCCGACCATGAAT-3'	<i>Sph</i> I <i>Hind</i> III	58	525
NCLIV_004430-C	F 5'- <i>gcatgc</i> AATTCCTCTAACTGCTCGATTG-3' R 5'- <i>aagctt</i> GTTAGCCTTGGAACAGTCC-3'	<i>Sph</i> I <i>Hind</i> III	59	471
NCLIV_038900	F 5'- <i>gcatgc</i> TCGGAGTTGTATTGAAGAAGG-3' R 5'- <i>cccggg</i> CGTGTGTTTGGTATTCCGTCG-3'	<i>Sph</i> I <i>Xma</i> I	56	582
NCLIV_053310	F 5'- <i>gcatgc</i> GAAGACGACGTAGTTGTCCTG-3' R 5'- <i>cccggg</i> CATTAAGAAGGGAAGAGCCCC-3'	<i>Sph</i> I <i>Xma</i> I	57	738
NCLIV_019580	F 5'- <i>gcatgc</i> GTGGAAGGGAAGAACTGG-3' R 5'- <i>aagctt</i> AGTTTTGTTCTTCTGCGTATCCG-3'	<i>Sph</i> I <i>Hind</i> III	58	345
NCLIV_023620	F 5'- <i>gcatgc</i> CAGGCCTCGAGAAATCTGGAAC-3' R 5'- <i>aagctt</i> GATGGGGCAGTCTTGCTTGTGG-3'	<i>Sph</i> I <i>Hind</i> III	58	390
NCLIV_040495-A*	F 5'- <i>gcatgc</i> AAGAGAATAGATGTTGTTAGCAC-3' R 5'- <i>cccggg</i> AGATGATCCTCTCGACAAGTC-3'	<i>Sph</i> I <i>Xma</i> I	58	606
NCLIV_040495-B	F 5'- <i>ggtacc</i> ATGACGTACACTCTCAATGTTTCCT-3' R 5'- <i>aagctt</i> GCTATGCGGACACGTAACCTC-3'	<i>Kpn</i> I <i>Hind</i> III	56	1,167
NCLIV_040495-C	F 5'- <i>gcatgc</i> CACACTACCCTCGATAATGGAGAG-3' R 5'- <i>aagctt</i> GGCAGGGTTTCTCTGGCG-3'	<i>Sph</i> I <i>Hind</i> III	58	603
NCLIV_005760	F 5'- <i>gcatgc</i> CCTGAACTGAAGAGCCAGC-3' R 5'- <i>aagctt</i> TGCAATACCTGCACCAGTTTC-3'	<i>Sph</i> I <i>Hind</i> III	59	960

Table 2.3 – Specific intra-sequence primers designed, restriction sites added at the 5' end of each primer, optimum annealing temperatures (Ta) and predicted amplicon sizes. Gene regions that were successfully expressed as recombinant proteins are shaded in yellow.

* Originally included within the gene denominated NCLIV_040495 (SRS-domain containing protein), the selected region NCLIV_040495-A was subsequently separated from downstream gene, based on expression, as a result of resequencing and reannotation (Ramaprasad *et al.*, 2015). This sequence is currently part of the sequence defined TPA: DNA polymerase, related [*Neospora caninum* Liverpool], accession number: CEL68278 (source GenBank: www.ncbi.nlm.nih.gov/genbank/).

2.2.4 Cloning

2.2.4.1 Cloning of the target sequences into the pGEM®-T Easy vector

The purified target amplicons were directly ligated into the linearised, high copy number pGEM®-T Easy Vector System (Promega, Madison, WI, USA) following the manufacturer's protocol (Appendix II).

After ligation, carried out as specified by the pGEM-T easy vector manufacturer, JM109 competent *E. coli* (Promega, Madison, WI, USA) were transformed with the vector-insert construct and screened using LB/ampicillin/IPTG/X-Gal made by adding 100 µg/ml ampicillin to melted LB-agarose and by spreading 100 µl IPTG 100 mM and 20 µl X-Gal 50 mg/ml on the surface of LB/ampicillin plates. Containing the β-galactosidase enzyme gene, the pGEM-T Easy plasmid allows blue/white colony screening. When insertion of DNA is successful the β-galactosidase enzyme gene is disrupted, therefore the enzyme cannot metabolise X-Gal into its normal product that confers blue colouration to the colony. White colonies should, theoretically, contain only vectors with the inserted DNA fragments of interest.

The presence or absence of the relevant insert was confirmed by colony PCR on white colonies. Identical PCR protocol and conditions described above were used substituting the DNA template with 5 µl DNase/RNase free water in which a small pick from a single bacterial colony was suspended. Colony PCR was carried out on six white colonies for each plate.

PCR positive colonies were then propagated by inoculation of 10 ml L-Broth containing 100 µg/ml ampicillin and incubated at 37°C in a shaking incubator (200 rpm) overnight. From overnight cultures, glycerol stocks of the transformed JM109 *E. coli* were prepared by adding 200 µl glycerol to 800 µl bacterial suspension and kept at -80°C for long-term storage of the plasmids. Additionally, plasmid DNA was isolated from aliquots of the overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands) and sequenced using the sequencing primers SP6-promoter and T7-promoter (Eurofins Genomics, Ebersberg, Germany) in order to verify the correctness of the DNA insert.

2.2.4.2 Cloning target sequences into the pQE-30 vector

QiaExpress® pQE vectors (Qiagen, Venlo, The Netherlands) (Appendix II) are used to express polyhistidine-tagged (6×His) proteins that can be easily purified through immobilised-metal affinity chromatography (IMAC). The 6×His-tag permits the tight, yet reversible, binding of the protein to metal chelating surfaces such as those of the HisPur™Ni-NTA (Thermo Fisher Scientific, Waltham, MA, USA) constituted by nitrilotriacetic acid (NTA) covalently modified to display divalent nickel ions (Ni²⁺). All the sequences of interest maintained an open reading frame when inserted into the pQE-30 vector; therefore, pQE-30 was chosen as cloning vector.

Double digestions using relevant restriction enzymes (New England BioLabs, Ipswich, MA, USA) (Table 2.3) were carried out in order to cut the target sequences out of the pGEM-T Easy insert/vector constructs as well as to linearise the pQE-30 vector. The enzymatic reactions were set up in CutSmart™ Buffer (New England BioLabs, Ipswich, MA, USA) and incubated at 37°C for 1.5 hours. After 45 min of incubation, thermosensitive shrimp alkaline phosphatase (TSAP) (Promega, Madison, WI, USA) was added to the digested pQE-30 in order to prevent recircularisation. Digests were electrophoresed on 1% (w/v) agarose/TAE gel incorporating Gel Red™ (Biotum, Hayward, CA, USA) and visualised under UV light. Bands corresponding to the sequences of interest and the linearised pQE-30 were excised from the gel. The DNA was isolated using the QIAquick® Gel Extraction Kit (Qiagen, Venlo, the Netherlands). Following ligation of the DNA inserts into the pQE-30 vector, carried out as specified by the manufacturer, JM109 competent *E. coli* were transformed with the insert/vector construct. Transformed bacteria were plated onto LB agar plates containing ampicillin (100 µg/ml) and incubated at 37°C, 5% CO₂ overnight. Single colonies were then screened by colony PCR as described previously. PCR positive colonies were propagated by overnight culture in L-Broth/ampicillin (100 µg/ml) and glycerol stocks made for long-term storage. Plasmid DNA was isolated and sequenced using the pQE vector primer 5' (Eurofins Genomics, Ebersberg, Germany) as indicated by the manufacturer of the expression system.

Cloning into pQE-30 was achieved for all target sequences listed in Table 2.3.

2.2.5 Expression of recombinant proteins

2.2.5.1 Transformation of M15 [pREP4] *E. coli*

M15 [pREP4] is the recommended *E. coli* strain for the expression of 6×His-tagged proteins using pQE vectors. The low copy number plasmid pREP4, which confers kanamycin resistance, constitutively expresses the *lacI* gene that allows high level production of the lac repression protein required for regulating recombinant protein expression.

M15 [pREP4] *E. coli* were transformed with the target DNA fragment/pQE-30 vector constructs. Transformed bacteria were plated onto LB/ampicillin (100 µg/ml) /kanamycin (25 µg/ml) agar plates and incubated at 37°C, 5% CO₂ overnight. Colony PCR was performed, as described above, to verify the transformation with the correct insert. M15 [pREP4] *E. coli* colonies presenting the correct DNA insert were cultured overnight in LB-Broth with ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Plasmid DNA was isolated for sequencing and glycerol stocks made and stored at -80°C until induction of protein expression.

2.2.5.2 Protein expression

Transformed M15 [pREP4] *E. coli* glycerol stocks were revived by overnight culture in LB-Broth with ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The following day, 5 ml of fresh pre-warmed culture broth aliquots were inoculated with 50 µl of overnight culture and incubated for 2 hours. All incubation steps were carried out at 37°C in an orbital shaking incubator at 200 rpm.

Un-induced 1 ml samples (un-induced control) were collected, centrifuged at 2,000×g, resuspended in 150µl PBS and 50µl NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) then stored at -20°C. Protein expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 2 mM. The bacterial culture was incubated for another 2 hours. Subsequently, a primary 1 ml induced sample (induced 1) was collected, pelleted, resuspended in 200 µl PBS and dyed with 66 µl NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) then stored at -20°C. The remaining culture was incubated for a further 2 hours. A secondary 1 ml induced sample (induced 2) was collected, pelleted, resuspended in

250 μ l PBS and dyed with 83 μ l NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA). The residual bacterial culture was centrifuged at 2,000 \times g for 5 min and the resulting bacterial pellet was stored at -80°C.

In order to confirm protein expression, un-induced and induced samples 1 and 2 were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions to allow optimal band visualisation. Briefly, samples were denatured by incubation at 99°C for 10 mins and loaded into NuPAGE® 4-12% Bis Tris 1 mm \times 15-well gels (Invitrogen, Carlsbad, CA, USA) and immersed in 200 ml NuPAGE® MES SDS Running Buffer (Invitrogen, Carlsbad, CA, USA). A protein standard (SeeBlue® Plus2 Pre-stained protein standard, Invitrogen, Carlsbad, CA, USA) was loaded for the determination of the molecular weight of protein bands and the gel was electrophoresed at 200 V for 40 min. The gel was washed three times for 5 min with dH₂O on an orbital shaker, stained with Simply Blue™ Safe Stain, Coomassie® G250 stain (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour, washed with dH₂O for another hour and visualised on ImageQuant™ (Ge Health Care Life Sciences, Little Chalfont, UK).

Molecular weights of the truncated polyhistidine-tagged recombinant proteins were predicted from the amino acid sequences of each target region using the Compute pI/Mw tool in ExPASy (http://www.web.expasy.org/compute_pi/).

2.2.6 Solubility assessment

Once protein expression was confirmed, the pellet obtained from the IPTG induced bacterial culture was resuspended in Binding Buffer 1 (without urea) (5mM NaH₂PO₄, 15mM Na₂HPO₄, and 500mM NaCl) (Appendix I). The bacterial suspension was disrupted by sonication for 10 s at 200-300 W 25% amplitude, cooled on ice for 20 s and sonicated for another 10 s at 200-300 W 25% amplitude. After centrifugation at 13,148 \times g, the supernatant, that should contain soluble proteins, was harvested and stored separately whereas the pellet, that should contain insoluble proteins, was resuspended in Binding Buffer 2 (urea) (8M urea, 200mM NaH₂PO₄, 200mM Na₂HPO₄, 500mM NaCl) (Appendix I) then centrifuged. SDS-PAGE loading dye was added to both supernatant and resuspended pellet and aliquots of the soluble

and insoluble fractions were analysed with SDS-PAGE in order to determine whether the recombinant proteins were soluble or insoluble.

2.2.7 Purification

Large scale propagation of *E. coli* M15 [pREP4] [pQE-30 (NCLIV_040495-A)], [pQE-30 (NCLIV_040495-C)] and [pQE-30 (NCLIV_004430-B)] was set up by inoculating 500 ml of pre-warmed L-Broth containing ampicillin (100 µg/ml) and Kanamycin (25 µg/ml) with 10 ml of overnight bacterial culture from revived glycerol stocks. After 2 hours incubation at 37°C in a shaking incubator (100 rpm), un-induced control samples were collected. Protein expression was then induced by adding IPTG, to a final concentration of 2 mM, to the bacterial cultures that were subsequently incubated for a further 5 hours. Induced samples were taken and the remaining culture was centrifuged at 5,000×g for 10 minutes. The resulting pelleted bacteria were resuspended in 5 ml Binding Buffer 1 (without urea) (Appendix I), sonicated and treated as previously described to produce soluble and insoluble proteins fractions. SDS-PAGE analysis was carried out to ensure the expression of recombinant proteins was successful. Since the three expressed recombinant proteins were insoluble, the insoluble proteins fractions were diluted in Binding Buffer 2 (8M urea) (Appendix I) and used for protein purification.

Hexahistidine-tagged tNcSRS44-A, tNcSRS44-C and tNcSRS12A-B proteins, encoded by target DNA fragments NCLIV_040495-A, NCLIV_040495-C, NCLIV_004430-B respectively, were purified by IMAC using HisPur™ Ni-NTA Spin Columns (Thermo Fisher Scientific, Waltham, MA, USA).

The procedure, was carried out as specified by the manufacturer. Briefly, spin columns were previously equilibrated at room temperature for 30 min and washed with 6 ml Equilibration buffer (Appendix I) removed by centrifugation. All centrifugation steps were carried out at 700×g for 2 min at room temperature. The protein extract was combined with an equal volume of Equilibration Buffer (200mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole in PBS [pH 7.4]) mixed thoroughly with the resin contained in the spin column, and incubated 30 min on an end-over-end mixer, at room temperature to allow binding of the polyhistidine-tagged protein to the resin. Spin

columns were then centrifuged to remove supernatant and washed three times with 6 ml Denaturing Wash Buffer (8M urea, 25mM imidazole in PBS [pH 7.4]) (Appendix I). After each centrifugation step the flow troughs were collected and stored separately. Elution was performed using Denaturing Elution Buffer (8M urea, 250mM imidazole in PBS [pH 7.4]) (Appendix I) in three sequential steps after each of which the eluted fraction was collected. The solutions resulting from the three washes and the three sequential elution steps were analysed with SDS-PAGE to ensure that no other proteins of bacterial origin had been eluted.

In order to verify that the visualised protein bands were actually the polyHistidine-tagged fusion proteins, a WB was carried out using monoclonal anti-polyHistidine peroxidase conjugated antibodies produced in mice (Sigma-Aldrich, Munich, Germany).

2.2.8 Dialysis

In order to decrease the concentration of urea and remove the imidazole that may interfere with downstream processing of the recombinant protein, the flow through obtained from the three elution steps were combined and dialysed.

Semi-permeable 10kDa dialysis tubing (Sigma-Aldrich, Munich, Germany) was boiled and cooled down in ultrapure dH₂O twice. After clamping and triple-knotting one end, the dialysis tubing was filled with the protein elution, knotted at the other end and suspended in 1 l of 4M urea dialysis buffer (Appendix I). The dialysis buffer was renewed after 4 hours, changed to 2M urea dialysis buffer overnight, and finally changed to 1M urea dialysis buffer (Appendix I) for 4 hours. At each changeover the protein solution was visually checked for the presence of precipitate, indicating if the protein had become insoluble. After dialysis to 1M urea the protein solution was removed from the dialysis tubing and diluted 1:2, 1:5, 1:10 and 1:100 in 1M urea/PBS and SDS-PAGE analysis carried out to check whether the recombinant protein was still present.

2.2.9 Immunogenicity assessment of recombinant proteins

In order to assess whether the produced recombinant proteins were able to be recognised by specific *N. caninum* IgGs, WBs using a panel of reference sera from experimentally and naturally *N. caninum* infected cattle were carried out.

2.2.9.1 *N. caninum* reference sera

Acute infection (tachyzoite positive)

Serum samples from Aberdeen Angus cross cattle experimentally infected with 5×10^8 *N. caninum* tachyzoites (NC-1 strain) administered subcutaneously during gestation (Benavides *et al.*, 2012) were regarded as representative of acute infection. All serum samples used were collected 21 days post-infection and tested ELISA positive with a commercial test (IDEXX *Neospora* Ab Test, IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer's cut-off values. A pool of 4 serum samples was tested by WB analysis.

Persistent infection (bradyzoite positive)

The serum of a naturally infected cow that repeatedly tested serologically negative, but from which *N. caninum* DNA was isolated from several tissues, was used as reference of persistent infection. Besides the absence of antibody titres detectable with commercial ELISAs, no cell-mediated responses to *N. caninum* tachyzoite antigens were observed in the lymphoproliferation assay (Benavides *et al.*, 2012). Of relevance, antibodies against the recombinant bradyzoite-specific rNcSAG4, but not against rNcGRA7, were detected in this cow indicating that the animal was likely to be persistently infected (Aguado-Martinez *et al.*, 2008).

In addition, sera from 4 Holstein-Friesian male calves, sampled 12 months after a primary subcutaneous challenge with 1×10^8 *N. caninum* tachyzoites (NC-1 strain), were also deemed to be representative of a persistent infection (Rocchi *et al.*, 2011). The sera of these animals also responded positively when tested with the bradyzoite-specific rNcSAG4 antigen.

N. caninum negative

Sera from negative control pregnant cattle used within a *N. caninum* experimental infection study, as well as sera from their foetuses collected and sampled after culling were used as negative reference sera (Benavides *et al.*, 2012). These animals always

tested seronegative and no DNA of the parasite was detected in their tissues. A pool of 4 adult serum samples and one individual foetal serum were tested separately in two WBs.

2.2.9.2 Western blot analysis

Recombinant tNcSRS44-A, tNcSRS44-B and tNcSRS12A-B proteins (encoded by NCLIV_040495-A, NCLIV_040495-C and NCLIV_004430-B respectively) were boiled and electrophoresed by SDS-PAGE under denaturing and reducing conditions as previously described (paragraph 2.2.5.2), then transferred onto nitrocellulose membranes (Amersham, Ge Health Care Life Sciences, Little Chalfont, UK) by electrophoresis at 30V for 1 hour. Denaturing and reducing conditions were the optimal choice as the insolubility of tNcSRS44-A, tNcSRS44-B and tNcSRS12A-B would have hindered any attempt in purifying them under native conditions (Bornhorst and Falke 2000).

After blocking with 4% w/v dried skimmed milk in PBS-T for 1 hour, nitrocellulose membranes were washed for 5 minutes in wash buffer (PBS 0.5% Tween® 80, 0.5 M NaCl) (Appendix I) three times and probed with reference sera diluted 1:200 in 2% w/v dried skimmed milk in PBS-T. Following 1 hour of incubation, the excess of serum was removed with three 5 min washes with wash buffer. Subsequently, horse-radish peroxidase-conjugated rabbit anti-bovine IgG (α BovIgG-HRP) (Sigma-Aldrich, Munich, Germany) was applied diluted 1:1,000 in PBS- T for 1 hour, membranes were rewashed as described previously and incubated for another 5 min with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualised on ImageQuant™ (Ge Health Care Life Sciences, Little Chalfont, UK). All incubation steps were performed at room temperature on an orbital shaker.

2.2.10 Specificity of recombinant antigens

Given the high predicted similarity between some of the recombinant *N. caninum* antigens and their homologous or orthologous *T. gondii* proteins, cross-reactivity was considered a likely occurrence. Recombinant *N. caninum*-derived proteins were tested for cross-reactivity by WB analysis, as previously described, using serum samples

from *T. gondii* seropositive cattle. Sera were collected from 5 Holstein Friesian calves orally infected with 1×10^6 *T. gondii* (M4 strain) oocysts, 28 days post-infection. Seroconversion was confirmed through *T. gondii* ELISA (ID Screen® Toxoplasmosis indirect, IDVet, Montpellier, France) and modified agglutination test (MAT) (Dubey and Desmonts 1987). Reciprocal *T. gondii* antibody titres were 800 in one calf and 1,600 in the other four calves when tested with MAT (Burrells *et al.*, in preparation). The sera were kindly provided by Dr Alison Burrells (Moredun Research Institute). In order to exclude concurrent infection with *N. caninum* or the presence of anti-*N. caninum* antibodies of colostral origin, all calves were *N. caninum* ELISA tested (ID Screen® *Neospora caninum* indirect multispecies, IDVet, Montpellier, France) before using them in the present study and they were all negative.

2.2.11 Quantification of recombinant proteins

The concentration of recombinant antigens dissolved in 1 M urea was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). According to the manufacturer's protocol, standard solutions of 1,000 µg/ml, 750 µg/ml, 500 µg/ml, 250 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 0 µg/ml bovine serum albumin (BSA) were prepared diluting the 2 mg/ml stock in 1M urea. These, as well as neat, 1:2, 1:5, 1:10 and 1:50 dilutions of the recombinant proteins were combined with the reagents provided and plated out in triplicate onto a 96-well flat bottomed microtitre plate. After 30 min incubation at 37°C, the OD₅₄₀ were measured on a plate reader (Dynex Technologies Headquarters, Chantilly, VA, USA). Recombinant proteins were diluted to a final concentration of 200 µg/ml and stored at -80°C until used as stock solution for the ELISA development.

2.2.12 ELISA

Only recombinant tNcSRS44-A and tNcSRS12A-B that showed immunoreactivity when tested with *N. caninum* positive tachyzoite and bradyzoite sera, in the WB, were used for the development of indirect ELISAs.

In order to establish the optimal working conditions, the 200 µg/ml stock solutions of each recombinant antigen were diluted 1:50 (0.4 µg/100µl), 1:100 (0.2 µg/100µl),

1:200 (0.1 µg/100µl), 1:400 (50 ng/100µl), 1:800 (25 ng/100µl) and 1:1,600 (12.5 ng/100µl) in coating buffer (0.1 M carbonate-bicarbonate buffer, [pH 9.6]). A checkerboard template was created to evaluate different concentrations of sera and secondary antibodies in relationship to different concentrations of coating antigen.

One hundred microliters of coating antigen dilution per well were added to 96 well High Binding M129B ELISA polystyrene microtitre plates (Greiner Bio-One GmbH, Kremsmünster, Austria) and incubated at 4°C overnight. Similarly, a polyhistidine-tagged NcSRS2 recombinant protein was used to coat part of each plate as a positive coating control.

Coated plates were washed six times with PBS containing 0.05% Tween® 20 (PBS-T) and blocked with 150 µl/well 4% w/v dried skimmed milk for 1h at 37°C. Plates were then washed twice with PBS-T and 100 µl cattle serum diluted 1:200 and 1:500 in 2% w/v dried skimmed milk were added to duplicate wells. Initially three reference sera were tested: *N. caninum* tachyzoite positive, putatively bradyzoite positive and a *N. caninum* seronegative control (see reference sera Paragraph 2.2.9.1). After incubation for 1h at 37°C, plates were washed six times with PBS-T and incubated for another hour with 100 µl/well horse-radish peroxidase-conjugated rabbit anti-bovine IgG (α BovIgG-HRP) (Sigma-Aldrich, Munich, Germany) diluted 1:1,000 or 1:2,000 in PBS-T. Plates were washed six times with PBS-T and 100 µl/well SureBlue™ TMB (3,3',5,5'-tetramethylbenzidine) Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA) were added. Reactions were left to develop for 5 min at room temperature in the dark and stopped by adding 100 µl/well 0.1 M HCl. Absorbance values were read at 450 nm (OD₄₅₀).

2.2.13 ELISA using Nickel-chelate plates

Immobilisation of 6×His-tagged proteins onto microtitre plates can also be achieved using plates coated with nitrilotriacetic acid covalently modified to display divalent nickel ions (Ni²⁺), based on the same principle that enables purification (Paragraph 2.2.4.2).

Nunc Immobilizer™ Nickel-Chelate 96 well plates (Thermo Fisher Scientific, Waltham, MA, USA) were firstly washed four times with approximately 200 µl of PBS-T per well. One hundred microliters of recombinant antigen diluted to 0.5 or 0.1

$\mu\text{g}/100\ \mu\text{l}$ in coupling buffer (0.01 M KCl) were then dispensed into the wells and the plates incubated at 4°C overnight. After incubation, plates were rinsed four times with PBS-T and used for carrying out ELISAs as described in Paragraph 2.2.12.

6 \times His-tagged NcSRS2 recombinant protein, previously synthesised, was used to coat part of each plate as a positive coating control to check the validity of the protocol used. Overnight incubation with only coupling buffer provided a negative coating control.

2.2.14 GST-fusion protein expression

Expression of tNcSRS44-A and tNcSRS12A-B was attempted using the GST-fusion system.

Glutathione-S-transferase (GST) from *Schistosoma japonicum* is a 26 kDa protein and is widely used as an affinity fusion partner for the purification of recombinant proteins. When positioned at the N-terminal end of recombinant proteins it aids the production of soluble products in *E. coli*. GST also protects the target protein from proteolytic degradation stabilising it into the soluble fraction.

Gene fragments similar to those encoding tNcSRS44-A and tNcSRS12A-B (NCLIV_040495 and NCLIV_004430 respectively) were identified depending on their suitability for insertion into the pGEX vectors (i.e. absence of intra-sequence restriction sites identical to those used for the double digestion during the excision stage). Gene-specific primers were designed (Table 2.4) and PCR amplification from *N. caninum* genomic DNA was carried out as previously described (Paragraph 2.2.3). Initial cloning into pGEM®-T Easy Vector System (Promega, Madison, WI, USA) was also performed as described above (Paragraph 2.2.4.1).

Table 2.4 – Gene-specific primers of NCLIV_040495 and NCLIV_004430 designed for cloning into the pGEX-5x-1 vector, restriction sites added at the 5' end of each primer, optimum annealing temperatures (Ta) and predicted amplicon sizes.

Gene ID-region	Primers	Restriction site added	Ta (°C)	Amplicon size (bp)
NCLIV_004430-D	F 5'- <i>gaattc</i> GCATTTGTTTGGACGGAGT-3' R 5'- <i>ctcgag</i> GGCAGTAGCTTCTGCCACG-3'	<i>EcoRI</i> <i>XhoI</i>	58	312
NCLIV_004430-E	F 5'- <i>gaattc</i> ACGGGCAGAACGCAACAC-3' R 5'- <i>ctcgag</i> TTCTTTGCTGGCAGTAGCTTC-3'	<i>EcoRI</i> <i>XhoI</i>	58	378
NCLIV_040495-D	F 5'- <i>gaattc</i> GTTCCAGCTTACCGTGGA-3' R 5'- <i>ctcgag</i> TCGCACATCGCAGGTATTCTC-3'	<i>EcoRI</i> <i>XhoI</i>	57	792
NCLIV_040495-E	F 5'- <i>gaattc</i> AAGCTGTGGATCAATGAGCC-3' R 5'- <i>ctcgag</i> CGCACCTTTCTCATTGGGTG-3'	<i>EcoRI</i> <i>XhoI</i>	58	573

2.2.14.1 Cloning into pGEX

GST-fusion protein expression constructs were generated by inserting the DNA fragments of interest into the multiple cloning site of the pGEX-5X-1 vector (Ge Health Care Life Sciences, Little Chalfont, UK). Double digestions using relevant restriction enzymes (Table 2.4) (New England BioLabs, Ipswich, MA, USA) were performed as previously described (Paragraph 2.2.4.2) and ligation of DNA inserts (amplicons shown in Table 2.4) into the pGEX-5X-1 vector carried as for manufacturer's specifications. BL21 (DE3) competent *E. coli* were transformed with the insert/vector construct. Transformed bacteria were plated onto LB agar plates containing ampicillin (100 µg/ml) and incubated at 37°C, 5% CO₂ overnight. Single colonies were then screened by colony PCR as described previously. PCR positive colonies were propagated by overnight culture in L-Broth/10mM MgCl₂/ampicillin(100 µg/ml)/0.2% glucose and glycerol stocks made for long-term storage. Plasmid DNA was isolated and sequenced using the pGEX forward and reverse primers (Eurofins Genomics, Ebersberg, Germany) as indicated by the manufacturer of the expression system. All gene targets listed in table 2.4 were successfully cloned into the pGEX-5X-1 vector.

2.2.14.2 Protein expression in *E. coli* strain BL21 (DE3)

Five ml aliquots of L-Broth/MgCl₂ 10mM /ampicillin (100 µg/ml)/0.2% glucose, pre-warmed at 37°C, were inoculated with 50 µl of transformed BL21 (DE3) *E. coli* previously revived in overnight culture. Cultures of BL21 (DE3) *E. coli* transformed with the pGEX-5X-1 plasmid vector without any insert were also set up. Induction of protein expression in these bacteria leads to the production of the 26 kDa GST-tag that served as a protein expression control.

Bacterial cultures were incubated at 37°C in shaking incubator with vigorous shaking (200 rpm) until the optical density measured at 600 nm (OD₆₀₀) reached 0.7 (about 2 hours) before collecting a 1 ml un-induced sample (un-induced control). Protein expression was then induced by adding IPTG at a final concentration of 2 mM. Induced samples 1 and 2 were collected after 2 and 4 hours' incubation respectively. Un-induced control, induced samples 1 and 2 were treated and analysed with SDS-PAGE, to verify GST-fusion protein expression, as previously described (Paragraph 2.2.5.2). The residual bacterial culture was centrifuged at 2,000×g for 5 min and the resulting bacterial pellet was stored at - 80°C.

2.3 Results

2.3.1 Expression of candidate genes using the pQE-30 vector

Three of the newly designed gene fragments, tNcSRS44-A (Figure 2.5 lanes 2 and 3) tNcSRS44-C (Figure 2.5 lanes 5 and 6) and tNcSRS12A-B (Figure 2.5 lanes 8 and 9) were expressed in transformed *E. coli* strain M15 [pREP4]. The truncated recombinant proteins produced were denominated based on the homologous or orthologous proteins expressed in *T. gondii* as suggested by Howe and Sibley (Howe and Sibley 1999).

Protein expression of the other candidate *N. caninum* gene regions, shown in Table 2.3, was not achieved due to difficulties during one of the phases leading to recombinant protein production (Table 2.5). Most of the problems occurred due to incorrect sequences inserted into the pQE-30 vector, observed after sequencing. Unsuccessful steps were repeated; in the case of repeated failure, target gene regions were abandoned and the study proceeded with the analysis of the successful candidates.

Table 2.5 – Progression of candidate gene regions towards recombinant protein production. ✓ – achieved step, ✗ – failed step. Gene regions that were successfully expressed as recombinant proteins are shaded in yellow.

Gene ID-region	PCR	Cloning into pGEMT-easy	Cloning into pQE-30	Correct gene fragment sequencing	M15 [pREP4] <i>E. coli</i> transformation	Protein expression
NCLIV_004430-A	✓	✓	✓	✓	✓	✗
NCLIV_004430-B (tNcSRS12A-B)	✓	✓	✓	✓	✓	✓
NCLIV_004430-C	✓	✓	✓	✗	✗	✗
NCLIV_038900	✓	✓	✓	✗	✗	✗
NCLIV_053310	✓	✓	✓	✗	✗	✗
NCLIV_019580	✓	✓	✓	✓	✓	✗
NCLIV_023620	✓	✓	✓	✗	✗	✗
NCLIV_040495-A (tNcSRS44-A)	✓	✓	✓	✓	✓	✓
NCLIV_040495-B	✓	✓	✓	✗	✗	✗
NCLIV_040495-C (tNcSRS44-C)	✓	✓	✓	✓	✓	✓
NCLIV_005760	✓	✓	✓	✓	✓	✗

Proteins **tNcSRS44-A** and **tNcSRS44-C** are regions of the *locus* CEL68278 (www.ncbi.nlm.nih.gov/genbank/) currently defined TPA (Third-Party Annotation) DNA polymerase, related [Neospora caninum Liverpool]. Originally submitted as **NCLIV_040495** and annotated as SAG-related sequence (SRS) domain containing protein (<http://www.toxodb.org>) (Reid *et al.*, 2012), the sequence underwent resequencing and reannotation with reclassification of the predicted product (Ramaprasad *et al.*, 2015) after it was selected and expressed as a candidate antigen within this work. tNcSRS44-A and tNcSRS44-C comprised 198 (positions 2 to 199) and 197 (positions 654 to 850) amino acids respectively within a 2,368 amino acids-long sequence (accession n. CEL68278) that showed high similarity with TgSRS44 (CST1) (accession n. KFG38379). Overall the proteins encoded by CEL68278 and KFG38379 showed 72% homology. In particular, tNcSRS44-A and tNcSRS44-C

displayed 74% (147/199) and 72% (141/197) amino acid identities respectively with the homologous regions in the *T. gondii* CST1 protein (Figure 2.4).

The other expressed antigen, **tNcSRS12A-B**, is a 170 (positions 23 to 192) amino acid long peptide belonging to the **NCLIV_004430** locus (accession n. XP003879997) sharing 47% (80/170) amino acid identity with its orthologue in the *T. gondii* genome TgSRS12A (Reid *et al.*, 2012) (Figure 2.4). After reannotation, the predicted product of NCLIV_004430 remained unchanged: SRS-domain containing protein.

The successful expression of tNcSRS44-A (Figure 2.5 lanes 2 and 3), tNcSRS44-C (Figure 2.5 lanes 5 and 6) and tNcSRS12-B (Figure 2.5 lanes 8 and 9) was confirmed by SDS-PAGE analysis. Protein bands, that were consistent with the predicted molecular weights for tNcSRS44-A, tNcSRS44-C and tNcSRS12-B (23, 25 and 19kDa respectively), were observed 2 (Figure 2.5 lanes 2, 5 and 8) and 4 hours (Figure 2.5 lanes 3, 6 and 9) after induction of *E. coli* M15 [pREP4] with IPTG. Such bands were not present in the samples before induction (Figure 2.5 lanes 1, 4 and 7).

(A)

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tNcSRS44-A      KRIDVVSTEGSQRFECVPVDSVLFPEQA-TGDSQSATVFVAVGADDSNCNIFDQPSRLGDLIP
TgSRS44         KKIEVIHGSEGARFECVPVESVLLPEQAMSGDIRAATVFVAADGSCNIFDPPSLGDEVVP
*.*:*: . . *****:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*:*
tNcSRS44-A      GASLQEELSVTADSATKAYTLVIPGLPSKVVKLCYVCVHTDNPEDSQVVVITVGRAGDGE
TgSRS44         GAYLQEELSVNADKAAKTYTLVIPGLPAKEVKLCYVCAHPDNPAGSQVTVITVRRSGDSE
** *****.*.*:*:*:*:*:*:*:*:*:*:*:******.*.*** .***.*** *:*:*.*
tNcSRS44-A      RGIPASAPVCKPSFSLEAWALGVRQPEVVDVTPAGQSVRFSCLSDATVFPHEAGARSAP
TgSRS44         NGNPASSPVCPSFSLEAWALGVRQPSVVDVAAPGQSVRFSCLSSTATELPSEAGARPAP
.* ***:*:*.******.******.******.******.******.*** :* *****.*
tNcSRS44-A      TQVYPVDGRGWCDLSRGSS-
TgSRS44         AQVYPVNGRGWCDLSKGPSS
:*:*:*:*:*:*:*:*:*.*.*

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Identities 147/199 (74%) Gaps 2/199 (1%)

(B)

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tNcSRS44-C      HTTLDNGEVTRKYTLTVEQLPGTPKRLCYLCVYNRDVLSNCGVMIDVASNRVGELEQNE
TgSRS44         ETAVDASEITTKYTFTVKQLPKTPKCLCYLCVYNRDVMNSCGVLIDVSSNSMTRLKSTSE
*:*:* .:*:* ***:*:*:*:* **.*****:*:*:*:*:*:*:*:*:*:*:*:*:*:*
tNcSRS44-C      DKIEPDQTVPTVELQCPPDYSAHTAATGLRLPEMELLVDSANRTVRFICPDDSSVFPEV
TgSRS44         EGIHTNPTVPTLELQCPPDYSAHTAATGLRLPEMELLVDSVNRRTARFVCPDDSSVFPEI
: * .: ***:*:*:*:*:*:*.******.******.******.******.*
tNcSRS44-C      DEVADPPSGKVYRVAGDSCDTRREETLLRDEVPGAELRKRILVEGGVVQRFYTFTAERLP
TgSRS44         DGEADPASGKVYRMTGDSCDTRREETAFLODEVGAELQRRTVVDGGVVQRYYIFKASRLP
* ***.******:*:******:*:*.******.*.* :*:******:* *.*.*
tNcSRS44-C      AEDRRLCYLCVRQRNPA
TgSRS44         EEDKKLCFLCVRQRNPA
**.******

```

Identities 141/197 (72%) Gaps 0/197 (0%)

(C)

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tNcSRS          AVCLDGVLLFSNGGAVAGDPVEGLLRRELS PDSSDAQGESGIAGVVATCTVASSGDAVTR
TgNcSRS12A     AVCLSGVLLFSSGNTIAAESFEGLLRRLSSGGNSNGAESVTPEIVATCPATSEGATAPV
*****.******.*.*:*:*:*.******:*:*..... ** .:*****:*.*...
tNcSRS          PATTLTSSSTLVATLECMGQSMKALPADAATVCVA-EATASKELNSSNCSIGDTSMGNP
TgNcSRS12A     PA-TLTLSSKSLTATVKCLDASHTAVPATATNVCVASDADVNGASDSESCIIGSSGGK
** *****.*:*.*:*:*:*.* *.*:*:* *:*.*** :* .. :*..* **..: *:*
tNcSRS          VKLQELLGTNDPVQWKAISSSDGKNQGESRMLSEASLPRTDKKFMVGCQ
TgNcSRS12A     VTLQSLLEATHPI SWTEKPASSDSQTGKERTLQLTETDLPRTDKTFFVGCQ
*.*.*.* .: *:*.* .:*..... *:*.* *:*:*:*:*.*:*:*

```

Identities 80/170 (47%) Gaps 1/170 (0%)

Figure 2.4 – Amino acid sequence alignment of (A) tNcSRS44-A (NCLIV_040495-A) (B) tNcSRS44-C (NCLIV_040495-A) with their homologous regions in TgSRS44 (TGME49_264660) and (C) tNcSRS12A-B (NCLIV_004430-B) with the orthologue TgSRS12A (TGME49_321490). (*) fully conserved residue, (:) conservation between groups of amino acids with strongly similar properties, (.) conservation between groups with weakly similar properties. Alignments were performed using the MUSCLE software (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

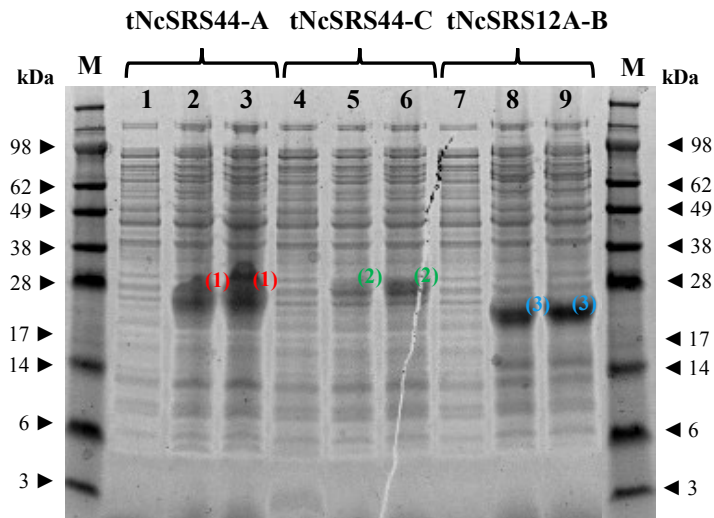


Figure 2.5 – SDS-PAGE showing protein expression of tNcSRS44-A (lanes 2 and 3), tNcSRS44-C (lanes 5 and 6), tNcSRS12A-B (lanes 8 and 9). M – molecular weight marker; lanes 1,4 and 7 – uninduced controls; lanes 2, 5 and 8 – IPTG induced (2 hours after induction); lanes 3, 6 and 9 – IPTG induced (4 hours after induction). **(1)** tNcSRS44-A (23 kDa), **(2)** tNcSRS44-C (25 kDa), **(3)** tNcSRS12A-B (19 kDa).

2.3.2 Solubility assessment

Proteins tNcSRS44-A, tNcSRS44-C and tNcSRS12A-B were insoluble in aqueous buffers that did not contain solubilising agents. SDS-PAGE analysis showed that all target proteins were detectable in the supernatant deriving from resuspension of sonicated bacterial pellets in Binding Buffer 1 containing urea (insoluble fraction), but not in the supernatant after resuspension in Binding Buffer 2 without urea (soluble fraction) (Figure 2.6).

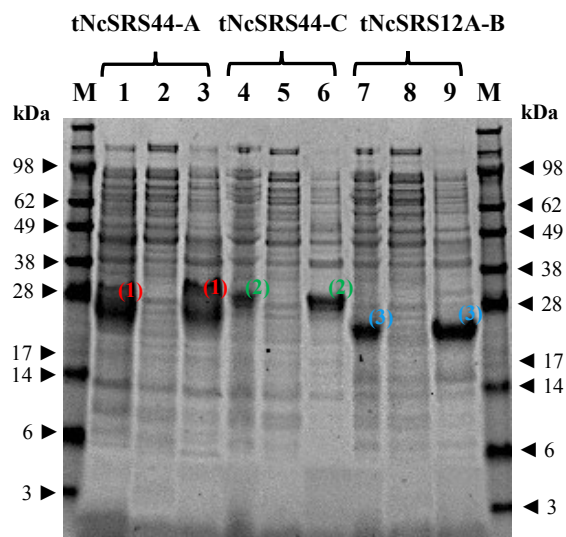


Figure 2.6 – SDS-PAGE showing the solubility/insolubility profiles of tNcSRS44-A, tNcSRS44-C and tNcSRS12A-B. All the truncated recombinant proteins were found within the insoluble fractions. M – molecular weight marker; lanes 1, 4 and 7 – induced bacterial cultures; lanes 2, 5 and 8 – soluble fractions (supernatant of bacteria resuspended in Binding Buffer without urea); lanes 3, 6 and 9 – insoluble fractions (supernatant of sonicated bacteria resuspended in Binding Buffer 8M urea). **(1)** tNcSRS44-A (23 kDa), **(2)** tNcSRS44-C (25 kDa), **(3)** tNcSRS12A-B (19 kDa).

2.3.3 Purification of expressed proteins

Purity of tNcSRS44-A (Figure 2.7), tNcSRS44-C and tNcSRS12A-B (Figure 2.8) was verified by SDS-PAGE analysis following IMAC purification. The absence of additional visible bands, indicated that the target recombinant proteins were free from major protein contaminations of bacterial origin.

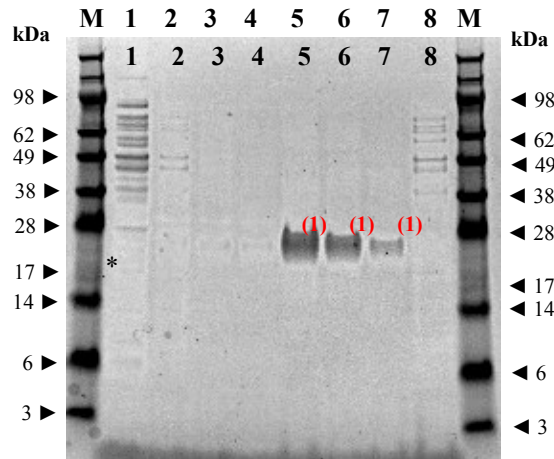


Figure 2.7 – SDS-PAGE showing purified tNcSRS44-A. Lane 1 – un-induced control; lanes 2, 3 and 4 – flow-through obtained from three sequential washes of the HisPur™ Ni-NTA Spin Columns; lanes 5, 6, 7 – flow-through obtained from three sequential elution steps; lane 8 – control supernatant before urea treatment (soluble fraction). M – molecular weight marker. **(1)** tNcSRS44-A (23 kDa).

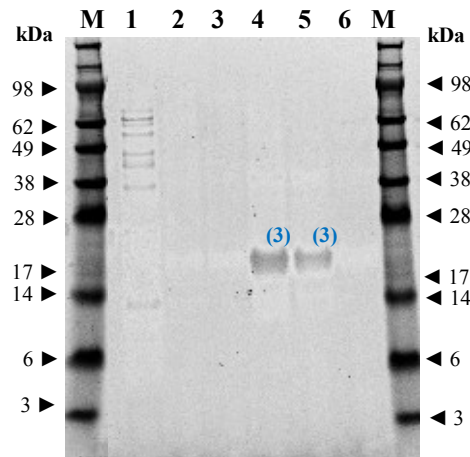


Figure 2.8 – SDS-PAGE showing purified tNcSRS12A-B. Lane 1 – un-induced control; lanes 2, 3 – flow-through obtained from two sequential washes of the HisPur™ Ni-NTA Spin Columns; lanes 4, 5 – flow-through obtained from two sequential elution steps; lane 6 – control supernatant before urea treatment (soluble fraction). M – molecular weight marker. **(3)** tNcSRS12A-B (19 kDa).

2.3.4 Immunoreactivity assessment of recombinant proteins

Proteins tNcSRS44-A and tNcSRS12A-B were found to be immunoreactive showing clearly visible bands in WBs (Figures 2.9, 2.10 B and 2.11 B) when assayed using serum samples from cattle considered persistently infected with *N. caninum* that tested negative with tachyzoite antigens-based serological tests (bradyzoite antigens positive animals). Weaker immunoreactivity was observed in the tNcSRS44-C-based WB as indicated by faint bands (Figure 2.9, lanes 5 and 6). Due to the weaker immunoreactivity of tNcSRS44-C, further analyses were carried out using tNcSRS44-A and tNcSRS12A-B only.

Reduced immunoreactivity of *N. caninum*-specific antibodies produced during acute infection (tachyzoite antigens positive animals) was observed for both tNcSRS44-A and tNcSRS12A-B as indicated by the low intensity bands in Figures 2.10 B and 2.11 B (lanes 2) with the latter showing a slightly higher band intensity.

As shown in Figures 2.10 B and 2.11 B low levels of antigen detection were observed when a pool of four sera from adult, putatively uninfected, cattle (lanes 3) was assayed in the tNcSRS44-A and tNcSRS12A-B-based WB analysis, respectively. Very low binding with specific antibodies, with hardly any visible bands, was observed when the serum of the foetus from an uninfected dam was used as a negative control serum (lanes 4).

Following dialysis of the recombinant proteins solubilised in urea, additional bands were observed in SDS-PAGE (Figures 2.10 A and 2.11 A) and consequently transferred to the WB (Figures 2.9, 2.10 B and 2.11 B) analysis. The molecular masses of these protein bands were consistent with those of dimers, trimers, tetramers and bigger multimers of tNcSRS44-A and tNcSRS12A-B that may have formed following the decrease of the urea concentration in the solubilising buffer following dialysis (Figures 2.9 and 2.10). In addition, the protein bands were confirmed to be 6×His-tagged proteins as they were recognised by monoclonal anti-polyHistidine antibodies (Figures 2.10 B and 2.11 B, lanes 5).

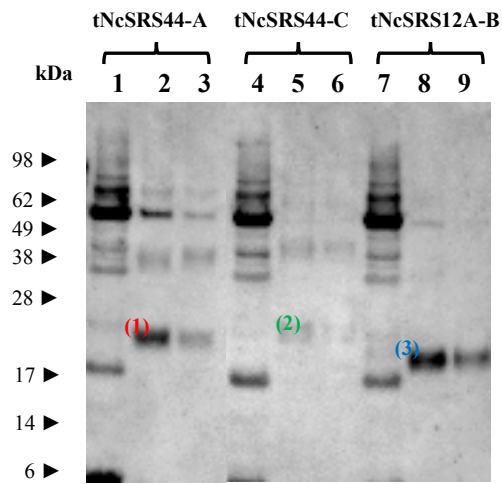


Figure 2.9 – Western blot showing the immunoreactivity of tNcSRS44-A (lanes 1, 2 and 3), tNcSRS44-C (lanes 4, 5 and 6) and tNcSRS12A-B (lanes 7, 8 and 9) when assayed with the serum of a persistently infected cow that tested negative with ELISAs based on tachyzoite antigens (bradyzoite positive sera). Lanes 1, 4 and 7 – control supernatant after urea solubilisation (insoluble fraction) and IMAC removal of target protein; lanes 2, 5 and 8 - recombinant protein obtained from the first elution step; lanes 3,6 and 9 - second elution step. **(1)** tNcSRS44-A (23 kDa), **(2)** tNcSRS44-C (25 kDa), **(3)** tNcSRS12A-B (19 kDa).

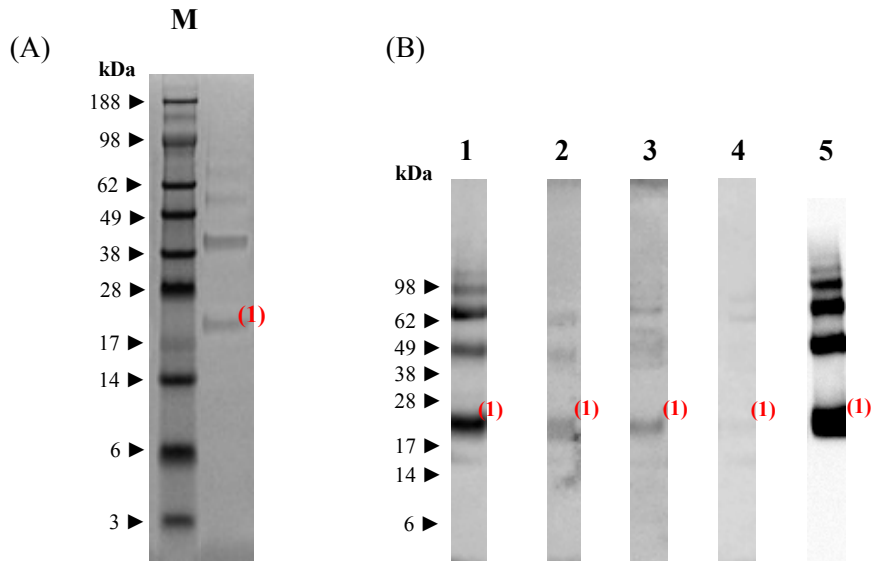


Figure 2.10 – SDS-PAGE analysis showing dialysed tNcSRS44-A (A) and Western blot based on tNcSRS44-A (B). 1 – Bradyzoite positive: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX Laboratories, Westbrook, ME, USA, and IDScreen® *Neospora caninum* indirect multi-species ELISA, IDVet, Montpellier, France); 2 – Tachyzoite positive: pool of 4 sera of acutely *N. caninum* infected cattle that tested seropositive with current commercial tests; 3 – Negative control 1: pool of 4 sera of putatively uninfected cattle that tested seronegative with current tests; 4 – Negative control 2: individual serum sample from the foetus of a seronegative cow; 5 – Anti-polyHistidine-peroxidase mouse monoclonal antibodies. M – molecular weight marker. (1) tNcSRS44-A (23 kDa).

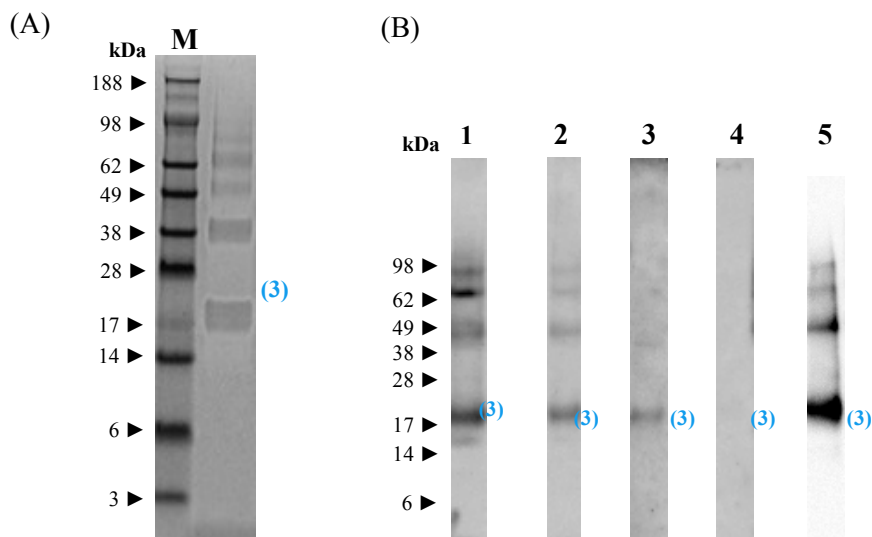


Figure 2.11 – SDS-PAGE analysis showing dialysed tNcSRS12A-B (A) and Western blot based on tNcSRS12A-B (B). 1 – Bradyzoite positive: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX Laboratories, Westbrook, ME, USA, and IDScreen® *Neospora*

caninum indirect multi-species ELISA, IDVet, Montpellier, France); 2 – Tachyzoite positive: pool of 4 sera of *N. caninum* acutely infected cattle that tested seropositive with current commercial tests; 3 – Negative control 1: pool of 4 sera of putatively uninfected cattle that tested seronegative with current tests; 4 – Negative control 2: individual serum sample from the foetus of a seronegative cow; 5 – Anti-polyHistidine-peroxidase mouse monoclonal antibodies. M – molecular weight marker. (3) tNcSRS12A-B (19 kDa).

2.3.5 Specificity of expressed antigens

Five *T. gondii* seropositive bovine serum samples were pooled and used to test the tNcSRS44-A and tNcSRS12-B antigens applying the same conditions (antigen concentration of 200 µg/ml and sera dilution 1:200) used for testing the *N. caninum* reference sera. In addition, the soluble fraction of *T. gondii* (strain M4) tachyzoite lysate was used to confirm the presence of *T. gondii*-specific antibodies in the *T. gondii* reference sera (Figure 2.12 C).

As shown in Figure 2.12 A and B, no detection of the recombinant *N. caninum* antigens by *T. gondii*-specific IgG antibodies (lanes 3) was observed. tNcSRS44-A and tNcSRS12A-B-based WBs were tested with the serum of a persistently infected cow (Figure 2.12 A and B, lanes 1) and with the pool of four *N. caninum* antibody negative sera (Figure 2.12 A and B, lanes 2) as positive and background negative control respectively.

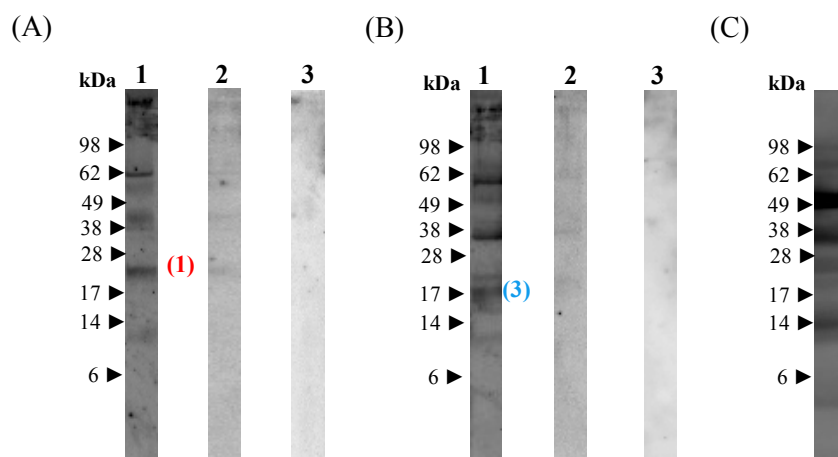


Figure 2.12 – Western blots showing the specificity of tNcSRS44-A (A) and tNcSRS12A-B (B). 1 – Positive control 1: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX

Laboratories, Westbrook, ME, USA, and IDScreen® *Neospora caninum* indirect multi-species ELISA, IDVet, Montpellier, France); 2 – Negative control: pool of 4 sera of putatively uninfected cattle that tested seronegative with current tests; 3 – *T. gondii* positive: pool of 5 sera from *T. gondii* experimentally infected and seropositive cattle. (1) tNcSRS44-A (23 kDa), (3) tNcSRS12A-B (19 kDa). (C) Positive control 2: *T. gondii* (strain M4) tachyzoite lysate-based Western blot tested with pooled *T. gondii* positive reference sera.

2.3.6 Development of ELISAs based on the recombinant antigens tNcSRS44-A and tNcSRS12A-B

2.3.6.1 Optimisation of ELISA variables

Recombinant tNcSRS44-A and tNcSRS12A-B were used to set up serum antibody ELISAs. Checkerboard titrations were used to determine the best combinations of antigen concentrations, reference sera dilutions and secondary antibody (α BovIgG-HRP) dilutions. OD₄₅₀ values obtained using two representative coating antigen concentrations (0.4 and 0.2 μ g/100 μ l) associated with two sera (1:50 and 1:100) and two conjugate (1:2,000 and 1:4,000) dilutions are shown in Figure 2.13 and 2.14. Subtracting the values obtained for the blank wells, the optical densities obtained for the reference sera from persistently (bradyzoite positive) and acutely (tachyzoite positive) infected *N. caninum* cattle were not indicative of specific antibody recognition when compared to those of the negative control. However, the overall validity of the protocol used was confirmed by the clearly positive results obtained testing a pool of sera from acutely infected animals with an in-house NcSRS2 ELISA that was simultaneously carried out on the same plate. Figures 2.13 and 2.14 show representative examples of several different repeated experiments.

In order to evaluate the possible non-adherence of the coating antigens to the polystyrene plates as a cause of ineffectiveness of the assays, checkerboard titrations analyses using monoclonal anti-polyHistidine peroxidase conjugated antibodies (diluted 1:100, 1:200, 1:500, 1:1,000, 1:2,000) were carried out to detect the presence of the coating antigens. Strongly positive OD₄₅₀ values were observed with all dilutions of the monoclonal antibodies tested demonstrating that the plates were suitably coated with the two novel recombinant antigens (data not shown).

2.3.6.2 ELISA using Nickel-Chelate plates

The use of Nunc Immobilizer™ Nickel-Chelate 96 well plates was also investigated to assess whether a more organised binding to the plate (6×His-tagged recombinant proteins should bind through the N-terminal end that displays the 6×His-tag) could enable the detection of specific antibodies. Nonetheless, no improvement of the assay was observed as shown in Figure 2.15. In comparison to the 6×His-tagged NcSRS2 recombinant antigen, that detected specific antibodies in the serum of acutely infected cattle, no such recognition was observed when the novel tNcSRS44-A and tNcSRS12A-B were used to coat the plates following the same protocol. Figure 2.15 shows a representative example of several different repeated experiments.

sera	α bovIgG HRP	tNcSRS44-A						NcSAG2				
		0.4 μ g/100 μ l				0.2 μ g/100 μ l		0.2 μ g/100 μ l				
		Blank 1	Neg 2	B+ 3	T+ 4	Neg 5	B+ 6	T+ 7	Neg 8	B+ 9	T+ 10	
1:50	1:2,000	A	0.039	0.146	0.084	0.148	0.104	0.063	0.117	0.422	0.192	2.274
		B	0.049	0.147	0.090	0.150	0.109	0.060	0.119	0.411	0.184	2.200
	1:4,000	C	0.038	0.112	0.072	0.119	0.090	0.060	0.096	0.334	0.136	1.739
		D	0.054	0.116	0.072	0.109	0.089	0.050	0.094	0.326	0.138	1.790
1:100	1:2,000	E	0.062	0.076	0.044	0.165	0.051	0.028	0.117	0.187	0.089	1.750
		F	0.039	0.058	0.048	0.164	0.068	0.026	0.117	0.190	0.089	1.740
	1:4,000	G	0.041	0.044	0.023	0.239	0.039	0.022	0.093	0.145	0.063	1.326
		H	0.040	0.044	0.027	0.148	0.038	0.024	0.099	0.153	0.064	1.445

Figure 2.13 – ELISA checkerboard analysis to determine the optimal combinations of coating antigen tNcSRS44-A (columns), serum dilution (1:50 and 1:100) and secondary antibody (α bovIgG HRP) (1:2,000 and 1:4,000) (rows). In this example, the coating antigen was tested at the concentration of 0.4 μ g/100 μ l and 0.2 μ g/100 μ l. Results are expressed in OD₄₅₀ values.

Neg – pool of 4 sera from *N. caninum* putatively uninfected cattle; B+ – serum from a *N. caninum* infected cow that tested negative with tachyzoite antigen-based commercial serological tests (bradyzoite positive); T+ – pool of 4 sera from *N. caninum* acutely infected cattle (tachyzoite positive). The same reference sera were tested with an in-house NcSRS2 ELISA that was run simultaneously to the novel antigens, on each plate.

sera	α bovIgG HRP	tNcSRS12A-B						NcSAG2				
		0.4 μ g/100 μ l				0.2 μ g/100 μ l		0.2 μ g/100 μ l				
		Blank 1	Neg 2	B+ 3	T+ 4	Neg 5	B+ 6	T+ 7	Neg 8	B+ 9	T+ 10	
1:50	1:2,000	A	0.041	0.184	0.112	0.101	0.125	0.092	0.074	0.419	0.225	2.304
		B	0.051	0.183	0.134	0.104	0.125	0.078	0.075	0.422	0.193	2.323
	1:4,000	C	0.045	0.144	0.095	0.072	0.096	0.065	0.057	0.319	0.152	1.811
		D	0.045	0.158	0.097	0.078	0.109	0.065	0.059	0.245	0.134	1.795
1:100	1:2,000	E	0.049	0.082	0.051	0.082	0.063	0.051	0.061	0.186	0.082	1.696
		F	0.046	0.083	0.05	0.07	0.067	0.037	0.058	0.167	0.131	1.719
	1:4,000	G	0.036	0.051	0.041	0.057	0.045	0.03	0.048	0.149	0.068	1.397
		H	0.035	0.061	0.037	0.064	0.045	0.031	0.047	0.151	0.07	1.363

Figure 2.14 – ELISA checkerboard analysis to determine the optimal combinations of coating antigen tNcSRS12A-B (columns), serum dilution (1:50 and 1:100) and secondary antibody (α bovIgG HRP) (1:2,000 and 1:4,000) (rows). In this example each coating antigens was tested at the concentration of 0.4 μ g/100 μ l and 0.2 μ g/100 μ l. Results are expressed in OD₄₅₀ values.

Neg – pool of 4 sera from *N. caninum* putatively uninfected cattle; B+ – serum from a *N. caninum* infected cow that tested negative with tachyzoite antigen-based commercial serological tests (bradyzoite positive); T+ – pool of 4 sera from *N. caninum* acutely infected cattle (tachyzoite positive). The same reference sera were tested with an in-house NcSRS2 ELISA that was run simultaneously to the novel antigens, on each plate.

sera	α bovIgG HRP	tNcSRS44-A				tNcSRS12A-B			NcSAG2			
		0.2 μ g/100 μ l				0.2 μ g/100 μ l			0.2 μ g/100 μ l			
		Blank 1	Neg 2	B+ 3	T+ 4	Neg 5	B+ 6	T+ 7	Neg 8	B+ 9	T+ 10	
1:50	1:2,000	A	0.075	0.149	0.105	0.199	0.117	0.107	0.113	0.179	0.135	1.651
		B	0.065	0.130	0.099	0.194	0.124	0.118	0.119	0.168	0.141	1.688
	1:4,000	C	0.064	0.119	0.093	0.133	0.107	0.094	0.085	0.130	0.094	1.049
		D	0.061	0.113	0.088	0.141	0.090	0.101	0.075	0.136	0.092	1.150
1:100	1:2,000	E	0.059	0.097	0.083	0.115	0.102	0.089	0.103	0.117	0.082	0.822
		F	0.060	0.093	0.085	0.106	0.121	0.091	0.099	0.112	0.083	0.860
	1:4,000	G	0.060	0.088	0.073	0.109	0.086	0.082	0.071	0.095	0.083	0.516
		H	0.058	0.087	0.073	0.083	0.077	0.070	0.069	0.092	0.092	0.487

Figure 2.15 – ELISA checkerboard analysis using Nunc Immobilizer™ Nickel-Chelate plates to determine the optimal combinations of serum dilution (1:50 and 1:100) and secondary antibody (α bovIgG HRP) (1:2,000 and 1:4,000) (rows). In this example the novel recombinant antigens were tested at the concentration of 0.2 μ g/100 μ l.

Neg – pool of 4 sera from *N. caninum* putatively uninfected cattle; B+ – serum from a *N. caninum* infected sera and conjugate (α bovIgG HRP) dilutions were used. Results are expressed in OD₄₅₀ values cow that tested negative with tachyzoite antigen-based commercial serological tests; T+ – pool of 4 sera from *N. caninum* acutely infected cattle. The same reference sera were tested with an in-house NcSRS2 ELISA (6xHistagged) that was run simultaneously to the novel antigens, on each plate.

2.3.7 Expression of candidate genes using the pGEX expression system

Since NcSRS44-A and NcSRS12A-B showed promising results within the immunoreactivity assessment but were not employable in the ELISA format, the production of GST-fusion proteins was considered as an alternative method to help the development of functional ELISAs. In order to achieve this, the pGEX expression system was used.

No visible protein expression was observed after induction of BL21 (DE3) *E.coli*, transformed with the pGEX-5X-1 in which the *N. caninum* gene regions NCLIV_004430-D and E (Figure 2.16, lanes 5 and 6) and NCLIV_040495-D and E (Figure 2.16, lanes 7 and 8) were inserted. SDS-PAGE analysis showed that, despite successful expression of the 26 kDa control GST tag (Figure 2.16, lanes 1 to 4), no bands of the predicted molecular masses of the four target GST-fusion proteins (36.2 (10.2+26), 38.9 (12.9+26), 53.9 (27.9+26) and 46.5 (20.5+26) kDa for tNcSRS12A-D and E and tNcSRS44-D and E respectively) were observed.

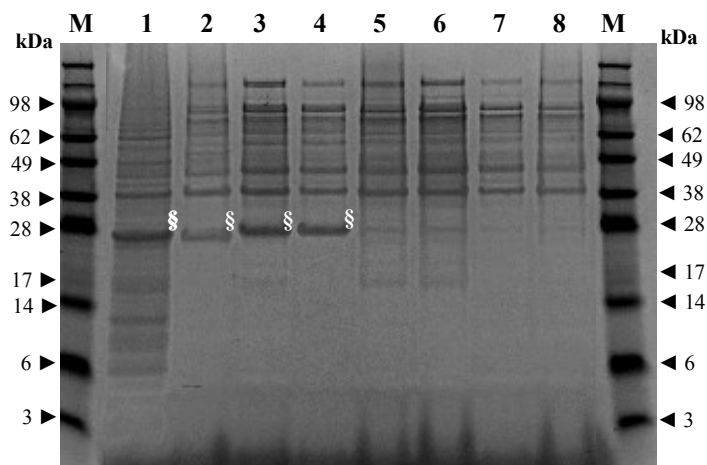


Figure 2.16 – SDS-PAGE showing failed expression of tNcSRS12A-D (lane 5), tNcSRS12A-E (lane 6), tNcSRS44-D (lane 7) and tNcSRS44-E (lane 8) as GST-fusion protein. Lane 1, 2, 3 and 4 show expression of the GST tag control for each target protein respectively. M – molecular weight marker; § – GST tag (26 kDa).

2.4 Discussion

2.4.1 Potential usefulness of tNcSRS44-A and tNcSRS12A-B for the identification of cattle persistently infected with *N. caninum*

One of the main challenges in the serological diagnosis of *N. caninum* in cattle is the detection of those animals that, despite harbouring the parasite, test serologically negative with current commercially available tests (Guido *et al.*, 2016). Given the epidemiological importance of these individuals, i.e. the ability of maintaining the infection over a number of generations in a herd through endogenous vertical transmission (Thurmond and Hietala 1997), improved diagnostics may help to control the disease more effectively.

Stage differentiation is a key event in the relationship between *N. caninum* parasites and the animal host (Hemphill *et al.*, 2006). Besides the consequences for the pathogenesis and epidemiology of neosporosis, the onset of persistent infections sustained by the bradyzoite stage may influence the diagnosis of the disease as suggested for the closely related parasite *T. gondii* (Gross *et al.*, 2004).

Due to the quiescent nature of the bradyzoite stage, bradyzoite-specific immune responses are, in general, expected to be of a lower intensity when compared to those elicited by the tachyzoites that multiply actively spreading throughout the body of the host. Since bradyzoites are enclosed within tissue cysts, exposure of bradyzoite antigens to the effector cells of the host immune system may be limited and recurrent rupture of tissue cysts may be necessary to induce detectable immune responses in persistently infected animals (Gross *et al.*, 2004).

N. caninum bradyzoite-specific antigens may be successfully employed for the detection of antibodies produced during persistent infection (Aguado-Martinez *et al.*, 2008). However, none of these bradyzoite-specific antigens have been made commercially available to date and serological tools that would enable the reliable identification of persistently infected animals are currently lacking in the market.

Accumulating evidence suggests that recombinant tachyzoite antigens derived from *N. caninum* show adequate specificity and sensitivity when used for the detection of specific antibodies produced against the parasite (Wapenaar *et al.*, 2007b). In addition,

recombinant proteins are easily produced in large quantities with standardised methods (Howe *et al.*, 2002).

In the present study, bacterial (*E. coli*) systems for the production of recombinant proteins were used. Alternatively, yeast (Pinheiro *et al.*, 2013), insect (Nishikawa *et al.*, 2004) or mammalian cell systems might have been used. The methylotrophic yeast *Pichia pastoris* would have had the advantage to promote post-translational modification and the expression of soluble recombinant proteins (Cereghino and Cregg, 1999). *P. pastoris* has been used previously to produce *N. caninum* recombinant antigen NcSRS2 (Pinheiro *et al.*, 2013). The same antigen was also effectively expressed using *Spodoptera frugiperda* (Sf9) cells co-transfected with recombinant baculovirus and *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Nishikawa *et al.*, 2001). In both studies, the recombinant surface antigens produced were successfully employed for the detection of *N. caninum*-specific antibodies in cattle. Furthermore, mammalian systems, such as Chinese hamster ovary (CHO) cells have proved to be effective for the expression of the *T. gondii* immunodominant antigen TgSAG1 (Kim *et al.*, 1994). In this study, the recombinant antigen showed conformational characteristics which were very similar to the native protein (Kim *et al.*, 1994). Nevertheless, most published studies in which recombinant *N. caninum* antigens were produced have used *E. coli*, since this expression system is reliable and cost-effective.

For these reasons, the identification and testing of novel *N. caninum* antigens that are expressed during the bradyzoite/tissue cyst stage may be an advantageous strategy for improving the detection of *N. caninum* persistently infected animals.

In the present work, a set of *N. caninum* DNA sequences putatively encoding antigens that are expressed by the bradyzoite stage of the parasite were selected. Sequence identification was based on inference from orthologous or homologous sequences in the *T. gondii* genome. The approach was dictated by the necessity of a simple and economical strategy that could capitalise on the existing genomic and transcriptomic information available for *T. gondii* and the genomic resources publicly available for *N. caninum*. Since the complete genome sequence is only available for the Liverpool strain of *N. caninum*, additional genome sequences would have helped to select target antigens for expression as recombinant proteins. The availability of multiple full

genome sequences of different strains of *N. caninum* would have enabled the selection of protein coding regions that are not polymorphic amongst strains, but are distinct from *T. gondii* sequences.

An alternative method to aid gene selection would be to experimentally assess whether the expression profile of each candidate gene was bradyzoite specific. This could have been done through the analysis of complementary-DNA (cDNA) generated from bradyzoite messenger-RNA (mRNA) obtained from either *in vivo* or *in vitro* models of stage conversion (Vonlaufen *et al.*, 2004).

Amongst the selected sequences, tNcSRS44-A and tNcSRS12A-B were expressed as recombinant proteins and showed immunoreactivity as they were recognised by specific IgGs in sera from persistently and to a lesser extent acutely infected cattle. In particular, tNcSRS44-A was recognised almost exclusively by sera from persistently infected animals whereas a slightly more intense band in the tNcSRS12A-B-based WB may suggest that the assay may also detect specific antibodies in serum samples from acutely infected cattle. The immunoreactivity profiles observed suggest that, similar to their *T. gondii* homologue and orthologue counterparts (TgNcSRS44 and TgSRS12A) NcSRS44-A and NcSRS12A-B may be expressed by the bradyzoite stage of *N. caninum*. In addition, the observation of a certain degree of recognition from sera from acutely infected animals may also suggest that NcSRS12A-B is not exclusively expressed by the bradyzoite stage and may be displayed also during the tachyzoite stage. However, further investigations are required to confirm stage-expression of these antigens. For example, quantitative RT-PCR for the assessment of gene expression could be applied on experimental models of persistent infection as previously reported for other bradyzoite-specific genes such as NcBSR4 (Risco-Castillo *et al.*, 2007) and NcSAG4 (Aguado-Martinez *et al.*, 2009) in mice models. Furthermore, immunolabelling assays using mono or polyclonal antibodies raised against tNcSRS44-A and tNcSRS12A-B could be carried out on *N. caninum* bradyzoites and tissue cysts (Kang *et al.*, 2008b, Kobayashi *et al.*, 2013) in order to assess the presence and localisation of the target proteins. These techniques, might also help to clarify the nature of tNcSRS44-A. Interestingly, the genic locus encoding tNcSRS44-A (accession n. CEL68278) was re-annotated after the fragment was cloned and expressed during this study. Formerly annotated as SRS-domain containing

protein, the sequence is now listed as predicted DNA-polymerase related protein. Surface localisation would suggest that tNcSRS44-A is more likely a surface antigen whereas a different (e.g. nuclear) localisation would provide indication of a possible relationship with DNA-polymerases.

In addition to the immunoreactivity with sera from persistently and acutely infected cattle, detectable reactivity was observed when serum samples from cattle deemed *N. caninum* negative were tested. Although lower compared to the responses of reference sera from infected animals, this finding may suggest either non-specific binding of antibodies to the proteins or the presence of the serum of one or more *N. caninum* persistently infected cows within the pool of sera tested. Cross-reactions with antigens from related apicomplexans, the animals may have encountered, cannot be completely ruled out.

In one study, insufficient purification of a recombinant dense granule (rNcGRA6) was reported to cause false-positive reactions (Jenkins *et al.*, 2005). Following purification, the SDS-PAGE page analysis of tNcSRS44-A (Figure 2.7) and tNcSRS12A-B (Figure 2.8) showed a good degree of purity of the recombinant proteins as indicated by the absence of additional protein bands. Therefore, the occurrence of false-positive reactions due to contamination was considered unlikely.

Due to the striking similarities between *N. caninum* and *T. gondii* the possibility of serological cross-reactivity between the two parasites should be considered when developing diagnostic tools. Several cross-reactive antigens, located either on the surfaces of the parasites or within organelles, have been reported and characterised using monoclonal antibodies produced against *N. caninum* (Liao *et al.*, 2005). In a proteomic study, using two-dimensional gel electrophoresis (2-DE) and immunoblotting with sera from rabbit immunised with *N. caninum* (strain KBA-2), a number of cross-reactive proteins such as heat shock protein 70 (HSP70), the protein disulphide isomerase (PDI), tubulin, actin α and β chains and enolase were identified (Lee *et al.*, 2005). In addition, ELISAs based on crude *N. caninum* tachyzoite antigens show different degrees of cross-reactivity with *T. gondii* when used for the serodiagnosis of canine neosporosis (Silva *et al.*, 2007). Interestingly, in one immunohistological study, antibodies against the bradyzoite-specific antigen of *T. gondii*, TgBAG5, cross-reacted with *N. caninum* bradyzoites (McAllister *et al.*, 1996).

In the present study, tNcSRS44-A and tNcSRS12A-B were not recognised when tested with serum samples from *T. gondii* experimentally infected cattle that had seroconverted. Ideally, cross-reactivity should have been tested using sera from *T. gondii* infected cattle with evidence of persistent infection; however, these were unavailable. Serum samples from sheep seropositive for *T. gondii* could also have been used to assess the cross-reactivity of the two novel *N. caninum* antigens described. Possible cross-reactivity with apicomplexans of the genus *Hammondia* (in particular *H. heydorni*) should also be considered as these parasites are structurally and antigenically similar to *N. caninum* with which they are closely related (Gondim *et al.*, 2015, Monteiro *et al.*, 2007). The information about serological cross-reactivity with *N. caninum* is scarce and bovine reference sera are difficult to obtain. In one study, a limited number of murine and ovine sera from *H. heydorni* experimentally infected animals did not show cross-reactivity with *N. caninum* surface antigens and total antigen extract when tested with IFAT and WB, respectively (Gondim *et al.*, 2015). Overall, the preliminary data of the immunoreactivity and serological specificity, as assessed with regards to *T. gondii*, indicated that tNcSRS44-A and tNcSRS12A-B may be useful for the detection of specific antibody titres in *N. caninum* persistently infected cattle. Further validation is required to assess the real diagnostic usefulness of the two antigens using adequate panels of well-characterised reference sera that should include a number of samples from cattle that are good representatives of persistent infection. However, such panels are difficult to create due to the current limitations in detecting *N. caninum* persistent infections, especially those occurring in naturally infected animals. The panel of serum samples used for validation in the present study included relatively few serum samples from cows which were considered to be persistently infected with *N. caninum*.

2.4.2 Towards a diagnostic ELISA for the identification of cattle persistently infected with *N. caninum*

The ultimate aim of this study was the development of a serum antibody ELISA to detect antibodies against the selected *N. caninum* bradyzoite-expressed antigens. Having a simple and relatively rapid execution, as well as objective interpretation, the ELISA represents the diagnostic technique of choice for high-throughput analysis.

Despite showing promising diagnostic potential in the WB format, tNcSRS44-A and tNcSRS12A-B were not effective in detecting specific *N. caninum* antibodies in the ELISA formats tested. The main differences between the two serological techniques were the physicochemical conditions in which the novel recombinant antigens were used. WBs were carried out with the antigens being blotted under denaturing and reducing conditions whereas in the ELISA such degree of denaturation and reduction would have been difficult to achieve.

Several studies reported the use of either reduced (i.e. the protein disulphide bonds are in the reduced state) or non-reduced (i.e. disulphide bonds are in the oxidised state) *N. caninum* immunodominant antigens in WBs (Atkinson *et al.*, 2000, Barta and Dubey 1992, Bjerkas *et al.*, 1994). Compared to reduced, non-reduced antigens showed stronger reactions suggesting that conformational epitopes may be predominantly involved in *N. caninum*-specific antibody responses (Dubey and Schares 2006). Based on these observations, the predicted amino acid sequences encoded by each of the candidate genes investigated in this study were screened for the presence of conformational epitopes; regions containing at least one of these were selected. Since conformational epitopes are discontinuous sets of amino acids brought into physical proximity by protein folding, correct folding is essential for determining the conformation that enables antibody binding. In order to conserve such structures purification and immunological assessment of the recombinant antigens should be carried out under native (non-reducing and non-denaturing) conditions.

Proteins tNcSRS44-A and tNcSRS12A-B were expressed as inclusion bodies in *E. coli* strain M15 p[REP4] as highlighted by their insolubility in aqueous solvents. Stored in the bacteria as inclusion bodies, recombinant proteins may fold assuming conformations that may differ significantly from the native protein (Baneyx and Mujacic 2004). In addition, purification of these proteins requires disruption of inclusion bodies and solubilisation using chaotropic agents, such as urea, that are denaturing. For this reason, denaturing and reducing conditions were an obligatory choice as the insoluble nature of tNcSRS44-A and tNcSRS12A-B would have hindered any attempt in purifying them under native conditions (Bornhorst and Falke 2000). Nevertheless, assessing the immunoreactivity of the recombinant proteins under denaturing and reducing conditions can reveal the presence of linear epitopes as

the proteins are unfolded. This was the case of tNcSRS44-A and tNcSRS12A-B in which the immunoreactivity observed suggests the presence of linear epitopes within their amino acid sequences. Unfortunately, such linear epitopes may have been inaccessible to serum antibodies when the recombinant proteins were used for the development of indirect ELISAs. One hypothesis to explain this was the occurrence of protein re-folding or aggregation that may have prevented the binding of specific antibodies to the epitopes. Protein folding may have occurred as a result of the different chemical environment the recombinant proteins were exposed to in the ELISAs compared to the WBs. Aggregation with the formation of polymers (mostly dimers, trimers and tetramers) was observed after the concentration of the chaotropic agent (urea) was decreased, by dialysis, from 8 M to 1 M. This phenomenon had no effect on the immunoreactivity of the recombinant proteins when used in the WBs (Figures 2.10 and 2.11); however, it may have interfered with the recombinant antigens when used to coat the ELISA plates.

In order to produce antigens that were similar to tNcSRS12A-B and tNcSRS44-A but characterised by water solubility, the expression of DNA fragments belonging to the NCLIV_004430 (NcSRS12A) and NCLIV_040495 (NcSRS44) as GST-fusion proteins was attempted. Despite several attempts, this option did not prove effective and GST-fusion protein synthesis was not achieved.

Assuming that structural rearrangements might have hidden the linear epitopes in tNcSRS44-A and tNcSRS12A-B, the possibility of developing ELISAs based on small (20 amino acids long) polypeptides was investigated. Synthetic polypeptides prepared from antigenic proteins may provide an alternative to the use of whole or bigger truncated proteins. In addition, they are stable, manufactured with highly standardised methods on a large scale and eliminate the risk of contamination with proteins of bacterial origin.

This approach included epitope mapping for the precise localisation of the epitopes within tNcSRS12A-B and tNcSRS44-A. Briefly, the amino acid sequences of the two target truncated proteins were divided into 20 residues-long polypeptides. Overlapping polypeptides including the last 10 and the first 10 residues of two adjacent sequences were also identified. This was done in order to increase the chances of including epitopes that may be located across the junctions of the peptides. From the short amino

acid sequences identified, synthetic polypeptides were produced as biotinylated molecules (ThinkPeptides, ProImmune, Oxford, UK) and their ability to bind specific *N. caninum* IgGs was assessed with dot blot.

In the dot blot analysis with reference cattle sera, 3 pairs of two contiguous peptides of tNcSRS44-A showed binding with *N. caninum*-specific antibodies in the serum of persistently infected cattle.

The development of polypeptide ELISAs work is currently in progress and it is carried out by Jackie Thomson (Moredun Research Institute) as a continuation of this work.

2.5 Conclusions

WBs based on the recombinant antigens tNcSRS12A-B and tNcSRS44-A may represent a supplementary diagnostic tool for bovine neosporosis. When associated with assays targeting tachyzoite-specific antibody responses, these tests could provide additional information particularly on those animals which tested antibody negative with tests based on tachyzoite antigens. Seropositivity with the tNcSRS12A-B and tNcSRS44-A WBs would suggest persistent infection whilst antibody negative results would enable to classify *N. caninum* seronegative cattle more confidently.

This method could be applied in those field conditions in which in-depth serological investigations are required and economically justifiable. For example, it would be beneficial within the selection of breeding stock in high genetic merit herds.

Further investigations are required for the development of functional ELISAs based on the antigens tNcSRS12A-B and tNcSRS44-A described in this study.

Chapter 3: Evaluation of antibody ELISA tests and estimation of *N. caninum* seroprevalence in British dairy cattle

3.1 Introduction

The serological diagnosis of *N. caninum* infection in live cattle plays a pivotal role within the overall management of the disease: from the investigation of abortion cases, in which the analysis of maternal antibody titres provides useful information on the involvement of the parasite in the foetal loss (McAllister 2016), through the assessment of the seroprevalence of the disease in specific cattle populations, to the implementation of control programmes that rely on the identification of infected animals and the selection of uninfected replacements (Reichel *et al.*, 2014).

Alongside the thorough investigation of abortion cases, the assessment of the serologic prevalence of bovine neosporosis provides useful insights on the distribution and impact of the disease, thus helping to shape effective control strategies both at the herd and the regional or national levels.

In the United Kingdom, the first assessment of *N. caninum* seroprevalence in non-aborting dairy cattle was provided by a case-control study conducted across England and Wales about 20 years ago (Davison *et al.*, 1999c). The study compared the serological status of aborting and non-aborting animals showing that the proportion of seropositive animals in the group of aborting cows (18%; 95%CI: 15-21%) ($n=633$) was significantly higher than in the control group (6%; 95%CI: 4%-8%) ($n=418$). The latter figure was the earliest estimate of the seroprevalence of bovine neosporosis in the country (Davison *et al.*, 1999c). During the same period, 17.1% of 4,295 cattle from 14 English dairy herds that had a history of *N. caninum* abortion were found to be seropositive (Davison *et al.*, 1999a). In a more recent longitudinal study, 12.9% of 15,736 cattle, which were tested at yearly intervals over a period of four years, were classified as *N. caninum* antibody positive at least once. Ninety-four percent of the 114 dairy and suckler herds included in the study had at least one seropositive cow (Woodbine *et al.*, 2008).

Another study investigating the association between *N. caninum* infection and the reproductive performance of 460 six-month-old dairy heifers reported a 7.2% seroprevalence. Seropositive heifers were more likely to suffer late embryonic or early foetal loss and abortion than seronegative heifers during their first (OR: 5.3, $p < 0.01$) and second pregnancy (OR: 6.0 $p < 0.001$) (Brickell *et al.*, 2010).

At the national level, bovine neosporosis is monitored by investigating bovine abortion cases (i.e. aborted fetuses with or without placenta usually with a corresponding serum sample from the aborting dam) that are submitted to the veterinary investigation centres as a mandatory requirement (APHA 2016). In 2014, *N. caninum* was diagnosed in about 25% of the bovine abortion cases that reached a diagnosis in England, Scotland and Wales (VIDA 2015).

Serosurveillance for bovine neosporosis is not normally performed and updated information on the seroprevalence of *N. caninum* in British cattle is currently lacking. Understandably, estimates of the seroprevalence vary depending on the study population, the geographical area of interest and the characteristics of the selected herds. However, the type of serological tests carried out as well as the cut-off levels used also affect the proportion of seropositive animals observed (Wapenaar *et al.*, 2007c).

Amongst the serological techniques that have been developed for the detection of *N. caninum*-specific antibodies in serum, plasma or milk (i.e. IFAT, ELISA, WB analysis, agglutination tests, and immunochromatographic tests), the enzyme-linked immunosorbent assay (ELISA) is the most frequently employed. Numerous variants and protocols have been reported (Dubey and Schares 2006). Although monoclonal and polyclonal antibodies have been used for the development of competitive inhibition ELISAs (CI-ELISAs) (Baszler *et al.*, 1996, Shin *et al.*, 2004), the vast majority of the tests that have been described to date, are based on the indirect format. Currently, most of the tests that are commercially available use sonicated or detergent-soluble total tachyzoite lysates as antigen preparation (Alvarez-Garcia *et al.*, 2013). However, assays based on native affinity-purified, ISCOMs-incorporated or recombinant tachyzoite antigens are also available (Alvarez-Garcia *et al.*, 2013).

N. caninum-specific antibody titres in cattle are known to fluctuate depending on the infection phase (i.e. acute, persistent and recrudescence infection) and the stage of

gestation (reviewed by Dubey and Schares 2006, Guido *et al.*, 2016). In some cases, tachyzoite-specific antibody levels decline below the detection limits of commercially available tests that are based on tachyzoite antigens so that previously serologically positive animals may become seronegative. This may occur in both aborting and non-aborting cows regardless of the stage of pregnancy (Nogareda *et al.*, 2007) and may result in a proportion of infected animals testing falsely negative with current commercial tachyzoite antigen-based diagnostics. Consequently, the seroprevalence may be underestimated.

Multiple testing with currently available tools may increase the chances of classifying individuals correctly (Ortega-Mora *et al.*, 2006). For example, cattle which test falsely negative may become antibody positive when retested following recrudescence of infection that elicit an increase in tachyzoite-specific antibody levels.

As previously discussed in Chapter 2, a few ELISAs were developed using antigens that are expressed by the bradyzoite stage of *N. caninum* in order to distinguish animals with persistent infection from those with acute or recrudescence infection (Aguado-Martinez *et al.*, 2008). However, none of these tools are commercially available to date.

Used in parallel with currently available assays, that are effective in detecting antibody titres against the tachyzoite stage, assays that target animals with persistent neosporosis may help to identify those cows that may not be identified by current tests (Guido *et al.*, 2016). This would also provide more accurate estimates of the seroprevalence of *N. caninum* in specific cattle populations.

In the present study, a commercial indirect ELISA (IDScreen® *Neospora caninum* indirect multi-species, IDVet, Montpellier, France) based on sonicated lysate of *N. caninum* tachyzoites and 5 indirect ELISAs, based on recombinant antigens (rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9), were used to assess the seroprevalence of bovine neosporosis in British dairy cattle.

NcSRS2 is an immunodominant surface antigen which is shared by both the tachyzoite and bradyzoite stages of *N. caninum* (Hemphill and Gottstein 1996). Nonetheless, its involvement in tachyzoite host cell invasion and the downregulation observed during the tachyzoite-to-bradyzoite stage conversion suggest a prevalent role at the tachyzoite stage (Vonlaufen *et al.*, 2004). In cattle, the detection of specific antibodies against

NcSRS2 has proven to be effective for the detection of infected animals (Ghalmi *et al.*, 2009, Nishikawa *et al.*, 2001, Pinheiro *et al.*, 2013, Schares *et al.*, 2000).

The tachyzoite dense granule-associated antigen NcGRA7 is highly immunogenic and is also expressed by both tachyzoite and bradyzoite stages (Hemphill *et al.*, 1998, Jenkins *et al.*, 1997, Lally *et al.*, 1996b). Several serological studies reported NcGRA7 as a valuable marker of acute infection in aborting and non-aborting cows (Aguado-Martinez *et al.*, 2009, Alvarez Garcia *et al.*, 2006, Huang *et al.*, 2007).

NcSAG4, NcBSR4 and NcSRS9 are *N. caninum* bradyzoite-specific antigens. NcSAG4 is expressed early in the bradyzoite conversion and was successfully employed for the detection of specific antibodies in cattle sera as well as foetal fluids (Fernandez-Garcia *et al.*, 2006, Risco-Castillo *et al.*, 2011). Used in parallel, antibody ELISAs based on recombinant NcSAG4 and NcGRA7 enabled the discrimination of cows with persistent, recrudescent or acute infection following a primary exposure to the parasite (Aguado-Martinez *et al.*, 2008). The detection of anti-NcSAG4 IgGs was correlated with persistent infection whereas NcGRA7-specific IgGs were linked to acute infection. Detectable antibodies against both antigens were considered suggestive of recrudescence or re-infection (Aguado-Martinez *et al.*, 2008).

Differently from NcSAG4, the expression of NcBSR4 and NcSRS9 is upregulated at a later stage following bradyzoite conversion (Risco-Castillo *et al.*, 2007, Risco-Castillo *et al.*, 2011). Since the expression of NcBSR4 and NcSRS9 was primarily observed in mature bradyzoites, it has been postulated that their exposure to the host immune system may be minimal and that rupture of tissue cysts would be required to elicit the production of specific antibodies during well-established persistent infections (Jimenez-Ruiz *et al.*, 2012). WB analysis showed that NcBSR4 and NcSRS9 can be used for the serodiagnosis of bovine neosporosis; however, only a small proportion of *N. caninum* naturally persistently infected cattle were recognised using both NcBSR4 and NcSRS9 antigens (Risco-Castillo *et al.*, 2011). Although NcBSR4 and NcSRS9 may be of some value for the identification of *N. caninum* persistently infected cattle, there is limited information to support their usefulness.

Setting appropriate cut-off levels and evaluating the characteristics and performances of the diagnostic tools is essential for their rational use (Alvarez-Garcia *et al.*, 2003, Jenkins *et al.*, 2002). In addition, the comparison between tests helps the selection of

those tools that are best suited for specific applications. This is particularly important within the serological diagnosis of bovine neosporosis in which a number of tests, especially ELISAs, are available and can be applied within different scenarios and for different purposes (Ortega-Mora *et al.*, 2006). For example, serological tests for the identification of infected cattle to be removed from the herd may require a higher sensitivity, thus lower cut-offs, than those used for the diagnosis of bovine abortions (Hall *et al.*, 2005, Schares *et al.*, 1999). Cut-off values favouring high sensitivity would also be required for purchase testing of individual animals to be introduced in herds that are free from the disease (Greiner and Gardner 2000, Roelandt *et al.*, 2015) or to exclude infected dams from embryo-transfer (Baillargeon *et al.*, 2001, Landmann *et al.*, 2002).

Within seroprevalence studies, accurate estimates require tests that are characterised by both high specificity and sensitivity.

In general, none of the techniques currently available for the detection of *N. caninum* infection can be regarded as a gold standard (Roelandt *et al.*, 2015). Historically, the immunofluorescence antibody test (IFAT) was considered the reference technique used in the evaluation of other serological tests. However, the interpretation of IFAT results is subjective and there is substantial inter-laboratory variation (Frössling *et al.*, 2003). In addition, as the serological test uses *N. caninum* tachyzoites, it targets exclusively antibodies produced against this stage of the parasite.

In the absence of routine *in vivo* techniques for the direct detection of the parasite which enable the accurate estimates of the characteristic of serological tests, and in the absence of large panels of fully and reliably characterised samples, no gold standard (e.g. Bayesian) analysis is strongly advised (Greiner and Gardner 2000, Frössling *et al.*, 2003, Dubey and Schares 2006). No gold standard methods are becoming increasingly employed for the assessment of serological tests used in seroprevalence studies when adequate panels of reference samples are lacking (Meredith *et al.*, 2015, Opsteegh *et al.*, 2010, Roelandt *et al.*, 2015). In the present study, a bi-modal mixture model fitted using Bayesian methods was used to discriminate between positive and negative results.

In summary, the main aims of this study were to:

1. Estimate the seroprevalence of *N. caninum* in British dairy cattle
2. Evaluate the test characteristics of one commercial ELISA and two experimental ELISAs based on recombinant *N. caninum* rNcSRS2 and rNcGRA7 antigens when applied to the determination of the seroprevalence within epidemiological studies
3. Evaluate the test characteristics of three experimental ELISAs based on the recombinant bradyzoite-specific antigens rNcSAG4, rNcBSR4 and rNcSRS9 when applied to the determination of the seroprevalence within epidemiological studies
4. Attempt to quantify the proportion of *N. caninum* infected animals that are not recognised by serological tests based on antigens highly expressed during acute infection.

3.2 Materials and methods

3.2.1 Study population

The study population consisted of adult female dairy cattle collected from herds across England, Scotland and Wales between November 2015 and February 2016. Cows were sampled as part of the metabolic profile blood testing that is periodically performed in a number of British dairy farms in order to assess the adequacy of the diet and prevent metabolic disorders at the herd level. These analyses were carried out by the Dairy Herd Health and Productivity Service (DHHPS), Royal (Dick) School of Veterinary Studies, the University of Edinburgh, which provided individual plasma samples and accompanying information.

Depending on the sampling regime for standard metabolic profile testing, the DHHPS requires samples from 17 randomly selected female adult cows per herd, subdivided as follow: 7 cows in early lactation (between 10-20 days post-calving), 5 cows in mid-lactation (80-120 days post-calving) and 5 dry cows (within 10 days from the expected calving date). However, the sample submitted did not always adhere to these recommendations.

The sample size was calculate using the WinPepi (version 11.53) software package (Abramson 2004). With a fixed number of 17 animals per cluster, an expected seroprevalence of 15%, an accepted error of 5%, a confidence level of 95% and the design effect (i.e. the loss of accuracy for using a cluster sampling design instead of a simple random sampling) set at 5.0 (Segura-Correa *et al.*, 2010), the necessary sample size was calculated at 969 cows from 57 herds.

A total of 1,037 individual bovine plasma samples from 61 herds were collected and tested.

Anonymised data for each sampled herd and individual animal were available through the DHHPS database. Selected herds were geographically located by the first three figures of their corresponding postal code. Individual information such as the productive/reproductive stage (early lactation, mid-lactation or dry period) with days in milk or predicted calving date as well as parity were provided.

The study was authorised by the ethical committee of the Royal (Dick) School of Veterinary Studies, the University of Edinburgh (VERC 32/15, 4/05/2015).

3.2.2 Study plasma samples

Individual whole blood samples collected into lithium heparin vacutainer tubes were centrifuged at 2,400×g for 10 min at 4°C soon after arrival at the DHHPS laboratory. From each individual sample, the plasma was carefully removed from the blood cell pellet by pipetting, aliquoted in 1 ml aliquots and stored at -20°C until testing.

3.2.3 Serological assays

3.2.3.1 Commercial *N. caninum* antibody ELISA

Presence of *N. caninum*-specific antibodies was initially determined using the commercial IDScreen® *Neospora caninum* indirect multi-species ELISA (IDVet, Montpellier, France). According to the manufacturer's specification, the test can be used to analyse serum, plasma or milk samples.

Testing was carried out as detailed by the manufacturer which supplied all reagents, buffer solutions and 96 microwell plates pre-coated with purified sonicated lysate of *N. caninum* tachyzoites. Briefly, 90 µl/well of Dilution Buffer was dispensed into the plate and 10 µl of test plasma, a positive and a negative control (*N. caninum* positive and negative freeze-dried bovine sera supplied by the manufacturer) were added in duplicate to the wells and incubated for 45 min at room temperature. After washing the plate three times with approximately 300 µl of Wash Solution, 100 µl of IgG-HRP conjugate was added to each well and the plate incubated for 30 min at room temperature. Wells were then washed three times with approximately 300 µl of Wash Solution, 100 µl of Substrate Solution was then added to each well and the plate incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 100 µl of Stop Solution. The optical densities at 450 nm (OD₄₅₀) were measured on a plate reader (Dynex Technologies, Chantilly, VA, USA). According to the manufacturer's instruction, results were deemed valid if the mean OD₄₅₀ of the positive control was >0.350 and the ratio of the mean OD₄₅₀ of the positive and the negative control duplicates was >3. The ELISA results were expressed as sample to positive control (S/P) percentage calculated using the formula: $S/P = (OD_{450} \text{ sample} - OD_{450} \text{ negative control}) / (OD_{450} \text{ positive control} - OD_{450} \text{ negative control}) \times 100$, where OD₄₅₀ is the mean value of the optical density of the duplicates.

3.2.3.2 Experimental *N. caninum* antibody ELISAs

Plasma was also tested using five experimental indirect ELISAs based on recombinant NcSRS2, NcGRA7, NcSAG4, NcBSR4 and NcSRS9 *N. caninum* antigens. All recombinant antigens were 6×His-tagged proteins produced using prokaryotic expression systems.

3.2.3.2.1 Recombinant antigens

Recombinant NcSRS2 (rNcSRS2) was previously produced in-house following a similar protocol as in Chapter 2 (Thomson, unpublished). Briefly, the NcSRS2 sequence was amplified by PCR from genomic *N. caninum* DNA (NC-1 strain) using the primers NcSRS2-forward (ggtaccGGTGTCTGGGTGCGTTCAAG) and NcSRS2-reverse (cccgggTCAGTACGCAAAGAT TGCCGTTGC). The amplicon obtained was then cloned into the pQE-31 vector system (Qiagen, Manchester, UK) and competent M15[pREP4] *E. coli* were transformed with the insert-vector construct. Following protein expression and verification the recombinant protein was purified as previously described (Chapter 2.2.7).

Recombinant NcGRA7 (rNcGRA7) (Alvarez-Garcia *et al.*, 2007), NcSAG4 (rNcSAG4) (Fernandez-Garcia *et al.*, 2006), NcBSR4 (rNcBSR4) (Risco-Castillo *et al.*, 2007) and NcSRS9 (rNcSRS9) (Risco-Castillo *et al.*, 2011) were kindly provided by Prof. Luís M. Ortega-Mora and Prof. Gema Alvarez-García (SALUVET, Complutense University of Madrid, Spain). Cloning, expression and purification of these antigens were carried out as described by Jimenez-Ruiz *et al.* (2012).

3.2.3.3 Reference bovine serum and plasma samples used for preliminary optimisation of the ELISA protocols

A small panel of reference sera from cattle deemed representative of acute ($n=12$) and persistent infection ($n=4$) as well as seronegative animals ($n=15$) was used for the preliminary intra-laboratory optimisation of the experimental ELISA protocols (Table 3.1).

Since rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 were to be applied to plasma specimens and plasma samples from experimentally infected cattle were unavailable, matched plasma and serum samples collected from naturally infected and

uninfected cows were compared. Matched plasma and serum samples were collected from aborting cows in which *N. caninum* was confirmed as causing agent ($n=5$), non-aborting cows from *N. caninum* problem herds ($n=5$) and cows from herds without a history of *N. caninum* abortions ($n=11$) (Table 3.1).

These samples were kindly provided by Dr Helen Carty (Scotland's Rural College, Auchincruive Veterinary Centre) and Dr Alastair Macrae (DHHPS, Royal (Dick) School of Veterinary Studies, the University of Edinburgh) and collected between May and September 2015.

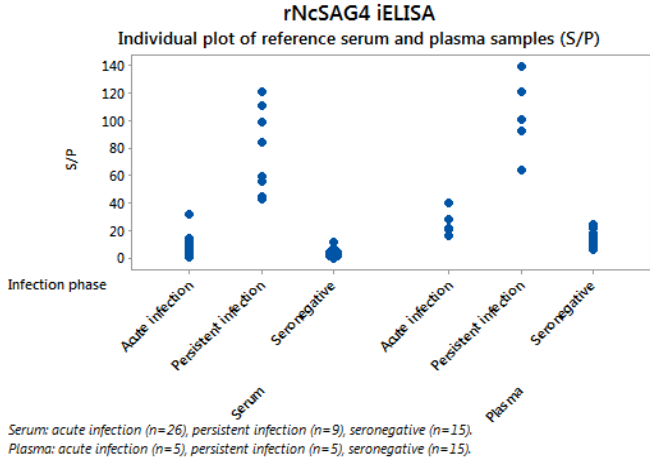
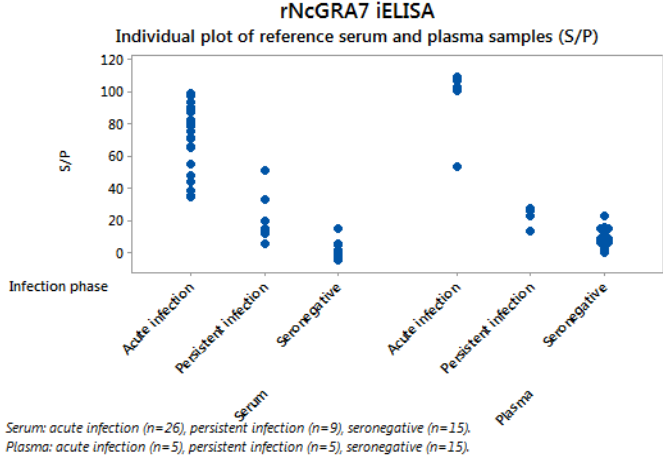
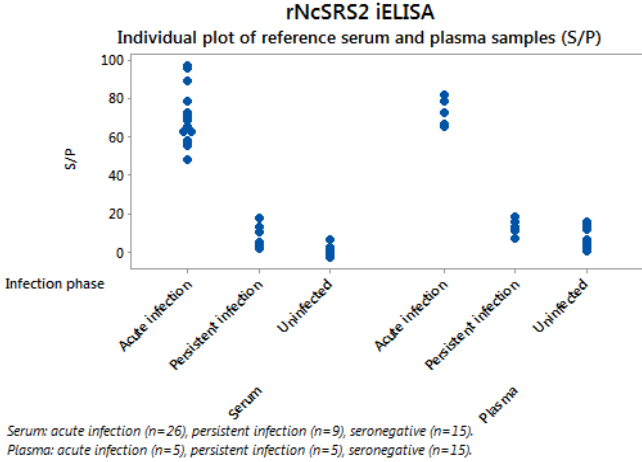
For the preliminary optimisation, the sera from known acutely infected and putatively persistently infected and uninfected cattle described in Chapter 2.2.9.1 were used as positive and negative controls. Briefly, a pool of 4 sera from cattle experimentally infected with 5×10^8 *N. caninum* tachyzoites (NC-1 strain) administered subcutaneously during gestation (Benavides *et al.*, 2012) was used as a positive control (acute infection). These sera, collected 21 days post-infection, tested positive with two commercial ELISAs: IDEXX *Neospora* Ab Test (IDEXX Laboratories, Westbrook, ME, USA) and IDScreen® *Neospora caninum* indirect multi-species ELISA (IDVet, Montpellier, France) according to the manufacturers' cut-off values. In addition, the serum from a naturally infected cow in which antibodies against the recombinant bradyzoite-specific rNcSAG4, but not against rNcGRA7, were detected, was used as a positive control for persistent infection (Aguado-Martinez *et al.*, 2008). Finally, a pool of 4 sera from adult cattle used as negative control animals within an experimental *N. caninum* infection study (Benavides *et al.*, 2012) was used as a negative control. These animals always tested seronegative with the two ELISAs mentioned above and no DNA of the parasite was detected in their tissues.

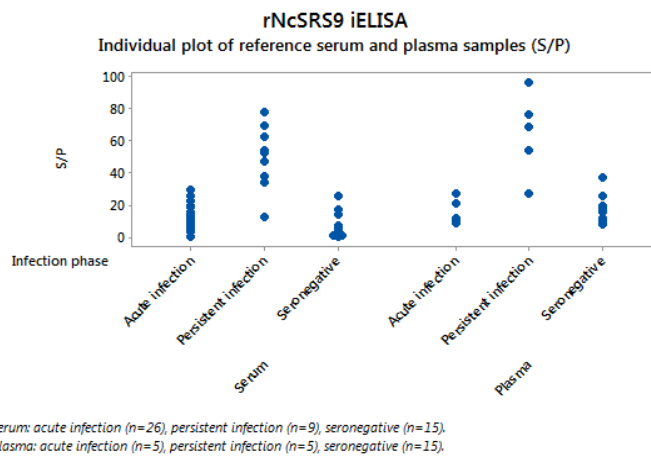
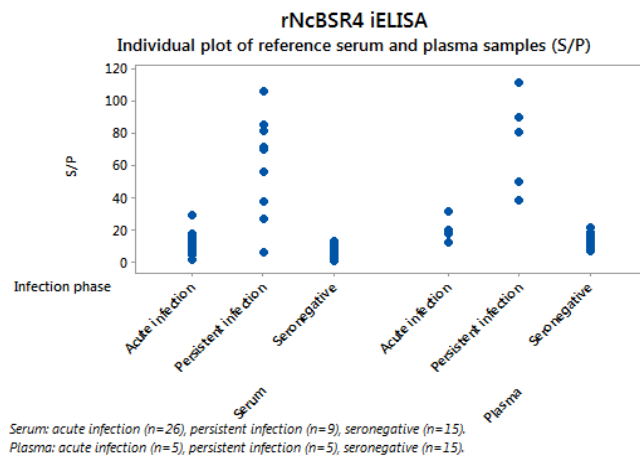
Individual results, expressed in S/P ratio, obtained for the reference serum and plasma samples using 5 experimental ELISAs are shown in Figure 3.1.

Table 3.1 – Origin and characteristics of the reference serum and plasma samples from *N. caninum* seropositive and seronegative cattle used for the optimisation of the rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs.

Experimental / Natural infection	Infection phase	Category	Isolate Dose/ Route	Sampling time	No. sera	No. plasma + serum	Reference/ Comment
Seropositive							
Experimental	Acute	Heifers infected at 210 days of gestation	NC-1/ 5×10 ⁸ / SC	21 DPI	6	0	Benavides <i>et al.</i> , 2012
	Acute	Heifers infected at 140 days of gestation	NC-1 1×10 ⁷ / SC	28 DPI	3	0	Shock <i>et al.</i> , unpublished IFAT titres = 1:1024
	Acute	Heifers infected at 210 days of gestation	NC-1 1×10 ⁷ / SC	28 DPI	3	0	Shock <i>et al.</i> , unpublished IFAT IgG titres = 1:512
	Persistent	Male calves	NC-1	360 DPI	4	0	Rocchi <i>et al.</i> , 2011
Natural	Acute	Aborting cows	-	After abortion	14	5	From 2 herds with ongoing enzootic abortions
	Persistent	Non-aborting cows	-	-	5	5	From 2 herds with history of enzootic abortions
Seronegative							
-	-	Adult cows (≥24 months)	-	-	11	11	From 4 herds with history of enzootic abortions
-	-	Male calves	-	-	2	0	Rocchi <i>et al.</i> , 2011
-	-	Foetuses from seronegative dam	-	-	2	0	Benavides <i>et al.</i> , 2012

Figure 3.1 – Preliminary optimisation of the ELISA protocols. Individual results, expressed as sample to positive percentage (S/P) ratio, obtained for the reference serum and plasma samples (described in Table 3.1) using the rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 experimental iELISAs.





3.2.3.4 ELISA protocols

The rNcSRS2 iELISA was developed and optimised in-house whereas the rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs were carried out as previously described (Aguado-Martinez *et al.*, 2008, Jimenez-Ruiz *et al.*, 2013) with minor modifications. Briefly, 96 well High Binding M129B polystyrene microtitre plates (Greiner Bio-One GmbH, Kremsmünster, Austria) were coated with 100 µl/well rNcSRS2 (0.1 µg/100µl), rNcGRA7 (0.2 µg/100µl), rNcSAG4 (0.1 µg/100µl), rNcBSR4 (0.2 µg/100µl) or rNcSRS9 (0.2 µg/100µl) diluted in coating buffer (0.1 M carbonate-bicarbonate buffer, [pH 9.6]) and incubated at 4°C overnight. After four washes with approximately 300 µl of PBS-T (PBS, 0.05% Tween® 20), plates were tapped dry and blocked with 150 µl/well of 4% w/v dried skimmed milk (rNcSRS2 iELISA), 4% w/v bovine serum albumin (BSA) (rNcGRA7, rNcBSR4 and rNcSRS9 iELISAs) or 2% horse serum (rNcSAG4 iELISA) diluted in PBS-T. The plates were incubated for 2h

at 37°C then washed twice and tapped dry. Test plasma samples, negative and positive controls were applied diluted 1:100 in 2% w/v dried skimmed milk (rNcSRS2 iELISA) or in the corresponding blocking buffer (rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs). After 1h incubation at 37°C, plates were washed four times and incubated for another hour at 37°C with 100 µl/well horse-radish peroxidase conjugated rabbit anti-bovine IgG (α BovIgG-HRP) (Sigma-Aldrich, Munich, Germany) diluted 1:2,000 (rNcSRS2 iELISA), 1:4,000 (rNcSAG4 and rNcGRA7 iELISAs) or 1:6,000 (rNcBSR4 and rNcSRS9 iELISAs) in PBS-T. After 1h incubation at 37°C, the conjugate was removed and the plates washed four times. One-hundred microliters/well of SureBlue™ TMB (3,3',5,5'-tetramethylbenzidine) Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA) was added, the reaction was left to develop for 5 min at room temperature in the dark and stopped by adding 100 µl/well of 0.1 M HCl. Absorbance values were read at 450 nm (OD₄₅₀). ELISA protocols are summarised in Table 3.2.

Controls and plasma samples were analysed in duplicate. Based on OD₄₅₀ values, plates were retested if duplicates for one or both controls had a coefficient of variation (CV=standard deviation of replicates/mean of replicates) above 20%. Similarly, individual plasma samples were retested if CV of the duplicates was >20%. Based on intra-laboratory optimisation, the results were deemed valid if the mean OD₄₅₀ of the positive controls was >0.8 and the ratio of the mean OD₄₅₀ of the positive control duplicates and the negative control duplicates was >5.

The ELISA results were expressed as sample to positive control percentages (S/P) calculated as described in Paragraph 3.2.3.1.

Table 3.2 – Summary of the ELISA protocols used in this study.

Test	Antigen	Reference*	Antigen (conc. µg/100µl)	Blocking buffer (conc. %)	Plasma dilution	Conjugate dilution
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	Purified <i>N. caninum</i> extract (sonicated tachyzoite lysate)	Alvarez-Garcia <i>et al.</i> , 2013	-	-	1:10	-
rNcSRS2 iELISA	Recombinant NcSRS2	-	0.1	Marvel (4%)	1:100	1:2,000
rNcGRA7 iELISA	Recombinant NcGRA7	Aguado- Martinez <i>et al.</i> , 2008	0.2	BSA (2%)	1:100	1:4,000
rNcSAG4 iELISA	Recombinant NcSAG4	Aguado- Martinez <i>et al.</i> , 2008	0.1	Horse serum (2%)	1:100	1:4,000
rNcBSR4 iELISA	Recombinant NcBSR4	Jiménez-Ruiz <i>et al.</i> , 2013	0.2	BSA (2%)	1:100	1:3,000
rNcSRS9 iELISA	Recombinant NcSRS9	Jiménez-Ruiz <i>et al.</i> , 2013	0.2	BSA (2%)	1:100	1:3,000

3.2.3.5 Assessment of serological cross-reactivity

In a previous study by Aguado-Martinez *et al.* (2008), the potential serological cross-reactivity of rNcGRA7 and rNcSAG4 with closely related Apicomplexan parasites was assessed by analysing sera from cattle infected with *S. cruzi*, *B. besnoiti* and *T. gondii*. No cross-reactivity was detected.

In the present study, the panel of *T. gondii* antibody positive sera from experimentally infected calves described in Chapter 2 (Paragraph 2.2.10) was used to check the specificity of the six tests employed. In addition, sera from six Holstein-Friesian, four weeks-old, calves which were experimentally infected with *Cryptosporidium parvum* were also tested. No cross-reactivity was observed.

3.2.4 Data analysis

3.2.4.1 Threshold, specificity and sensitivity determination

In the absence of a gold standard method for the identification of truly *N. caninum* seropositive and seronegative animals and adequate numbers of appropriate reference plasma samples from confirmed *N. caninum* infected and uninfected cattle, the

threshold for classifying the ELISA results, was determined, for each test, using a statistical method as described in Opsteegh *et al.* (2010) and Meredith *et al.* (2015).

Assuming that the study population consists of a mixture of seropositive and seronegative cattle, the frequency distribution of the observed ELISA results, in this case expressed as S/P percentages, should show two components that are both assumed to be normally distributed. These two normally distributed components of the study population should be characterised by different mean and standard deviation: a higher mean is assumed for the seropositive population and lower mean for the seronegative (Opsteegh *et al.*, 2010). Estimates for mean and standard deviation of the distributions representing negative and positive results are obtained by fitting a bi-modal normal distribution model to the observed S/P values. Bayesian Markov chain Monte Carlo (MCMC) methods, implemented using the JAGS software (Denwood 2013) interfaced using the 'runjags' package (Plummer 2016) in R Studio (version 0.99.902) were used to fit the bi-modal normal distribution model. The analysis was performed with the help and under the supervision of Dr Darren Shaw (Royal (Dick) School of Veterinary Studies, the University of Edinburgh) who kindly provided the R script which was applied as previously described (Meredith *et al.*, 2015). Nine chains with over-dispersed starting values were used and trace plots visually assessed to ensure convergence, non-converging chains were excluded. Optimum threshold values, defined as the highest total sensitivity and specificity of each test, were calculated from the posterior distributions generated as output of this simulation. For each iteration of parameter values, two cumulative distribution functions were used to calculate the probability of observing an S/P value above and below the given threshold for the negative and positive component of the distribution respectively.

Using the calculated threshold and distributions fitted to the observed data, median sensitivity and specificity were calculated for each test.

ELISA results obtained with each one of the 6 ELISAs used were dichotomised based on the calculated thresholds (cut-offs) so that each animal was scored positive or negative with each test.

3.2.4.2 Test agreement

Test agreement, expressed as Kappa statistic (κ), was calculated on pairs of tests using the “cohens.kappa” function interfaced using the ‘psych’ package in R Studio (version 0.99.902). The kappa coefficients were interpreted according to the guidelines outlined by Landis and Koch, where the strength of the kappa coefficients was defined as slight ($\kappa = 0.01-0.20$), fair ($\kappa = 0.21-0.40$), moderate ($\kappa = 0.41-0.60$), substantial ($\kappa = 0.61-0.80$) and almost perfect ($\kappa = 0.81-1.00$) (Landis and Koch 1977). Confidence intervals were reported alongside the Kappa-values.

3.2.4.3 Comparison of seroprevalence by productive/reproductive group

The binary response of outcome ELISA obtained with the IDVet iELISA (response variable) was modelled using generalised linear mixed-effect models with binomial errors fitted by maximum likelihood (Laplace approximation) to consider the variation in seroprevalence between the 3 different productive groups: early lactation, mid-lactation and dry cows.

The farm of origin was entered as a random effect to take into account farm-related epidemiological conditions, such as farm-specific infectious pressure, whereas the productive group (early, mid or dry) was added as fixed effect.

Biological assumptions, based on the evidence that reactivation of the parasite is more likely to occur towards the last trimester of pregnancy (i.e. when the majority of *N. caninum* abortion cases are observed), suggest an increased likelihood of infected cattle to test serologically positive during the dry period. Therefore, testing dry cows for *N. caninum*-specific antibodies is generally advised in practice.

In order to investigate whether there were statistically significant variations in the seroprevalence in cows tested during the dry period compared to early and mid-lactation, the group dry was entered in the model as reference group (intercept).

Regarding the lactation number, the cattle belonging to the study population for which the information was available were classified based on 6 levels (0, 1, 2, 3, 4, and >4). Lactation number was entered as a fixed effect in a univariate model and in association or in interaction with the productive/reproductive group in order to investigate its potential role as a confounder. The analysis was carried out using the “glmer” function available with the ‘lme4’ package in R Studio (version 0.99.902). Statistical

significance was determined at the level of $p < 0.05$. The data were modelled with the help and under the supervision of Dr Hanna Ensor (BioSS, Biomathematics and Statistics Scotland).

3.3 Results

3.3.1 Descriptive statistics of cattle data

A total of 1,037 plasma samples, collected from 61 British dairy farms, were tested for *N. caninum*-specific antibodies. The first three figures of the postal code were available for all farms. Only three postal codes were repeated twice; however, they identified different farms as indicated by the DHHPS customer identification numbers. Since the use of residual samples submitted for different diagnostic purposes was chosen for convenience, stratified sampling, to consider number and size of dairy farms in each region, was impractical. The South West and North West of England, regions with the highest number of dairy cattle, together with Scotland, were the areas where most of the sampled herds were located (Table 3.3). The geographical distribution of the herds tested is shown in Figure 3.2.

At the time of sampling, 406 (39.1%) cows were in early lactation (days in milk: mean \pm SD 18.7 ± 7.2 , median 15.00), 317 (30.5%) in mid-lactation (days in milk: mean \pm SD 104.2 ± 36.1 , median 97.00) and 314 (30.2%) during the dry period (days prior to expected calving date: mean \pm SD 11.4 ± 9.6 , median 7.00).

In most herds, the sample included 7 cows in early lactation, 5 in mid-lactation and 5 dry cows (41/61); however, in 9 herds the distribution early, mid or dry was 6-5-6 respectively, in 6 herds 6-6-5 and 5-5-7, 7-6-4, 8-5-4, 9-3-5 and 0-12-5 in the remaining 5 herds respectively.

The lactation number, that is indicative of parity and age, was reported for 952 out of the 1,037 (91.8%) cows and ranged between 0 (heifers due to calve for the first time) and 10 (mean \pm SD 2.67 ± 1.71 , median 2.00). All heifers belonged to the “dry cows” group representing 28.0% of the animals in this group.

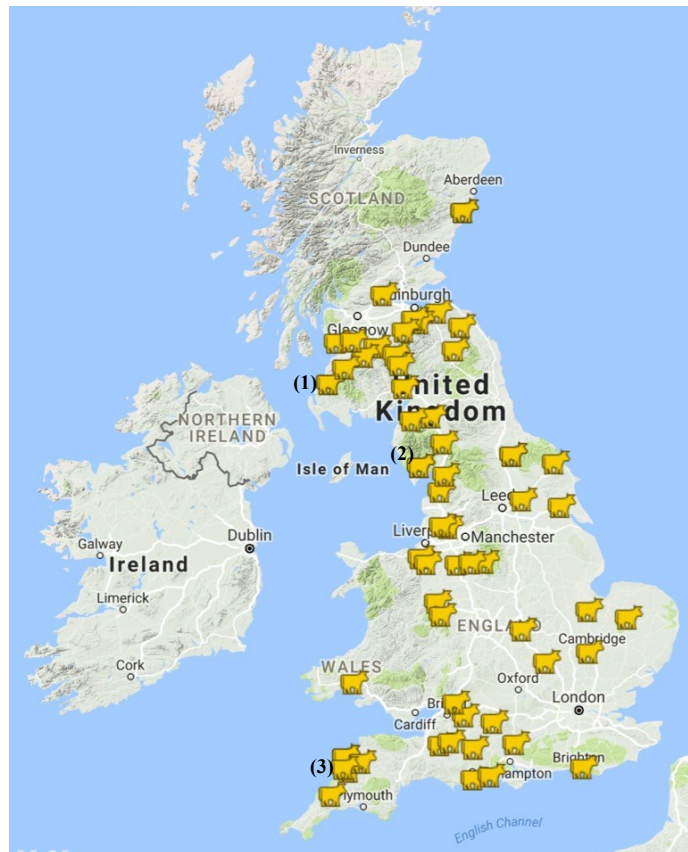


Figure 3.2 – Geographical distribution of the herds tested ($n=61$) based on the first three figures of the farm postcode (map generated using www.google.co.uk/maps). The postcodes (1) KA26, (2) LA12, (3) EX39 identify two distinct herds located within the same postcode area respectively.

Table 3.3 – Number and proportion of cattle sampled per region and size of the dairy cattle population in each geographical area of Great Britain.

Region	Samples (herds)	Proportion of samples per region (%)	Dairy cattle per region (AHDB Dairy, 2015)	Proportion of cattle per region (%)
Scotland	238 (14)	23.0	289,600	11.4
North East, Yorkshire and Humber	102 (6)	9.8	178,208	7.0
North West	204 (12)	19.7	495,406	19.5
East Midlands	51 (3)	4.9	140,698	5.5
West Midlands	34 (2)	3.3	292,886	11.5
Wales	34 (2)	3.3	246,331	9.7
East of England	51 (3)	4.9	31,021	1.2
South East	51 (3)	4.9	119,139	4.7
South West	272 (16)	26.2	751,770	29.5
Total	1037 (61)	100.0	2,545,059	100.0

3.3.2 Threshold determination and test characteristics

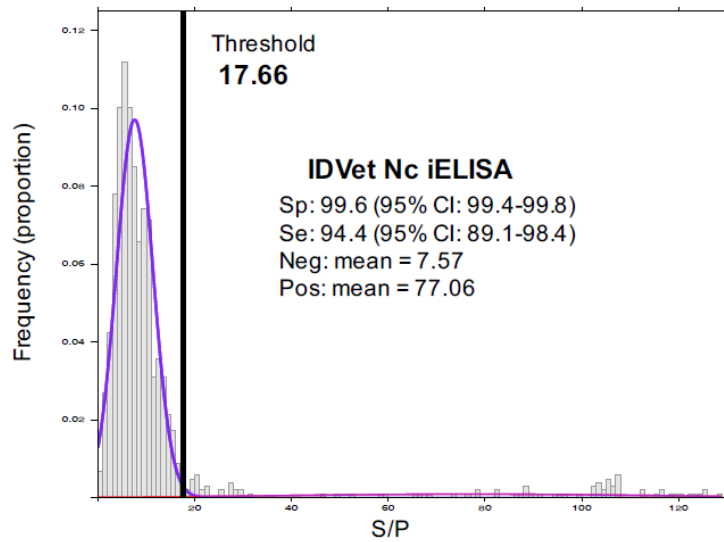
The frequency distribution of the ELISA outcome (S/P percentages) obtained analysing the 1,037 plasma samples with the ELISA tests based on immunodominant antigens considered to be markers of acute infection (IDVet, rNcSRS2 and rNcGRA7) showed two distinct populations of negative and positive animals (Figure 3.3 – A-C). Such distinction was not observed in the ELISA outcome frequency distribution of the ELISA tests based on bradyzoite-specific antigens (rNcSAG4, rNcBSR4 and rNcSRS9) in which the higher S/P values were not clearly separated from the population characterised by lower S/P values and were skewed forming a long “tail” instead of a separate component (Figure 3.3 – D-F). This lack of two distinct components was particularly evident for the rNcBSR4 and rNcSRS9 iELISAs.

The MCMC bi-modal normal mixture model converged upon two distinct distributions with median estimates representing the mean of the negative and positive population for each of the 6 antibody ELISAs used (Table 3.4). These distributions, that best described the two observed populations of negative and positive results, were fitted and drawn alongside the frequency distribution of the observed results (Figure 3.3).

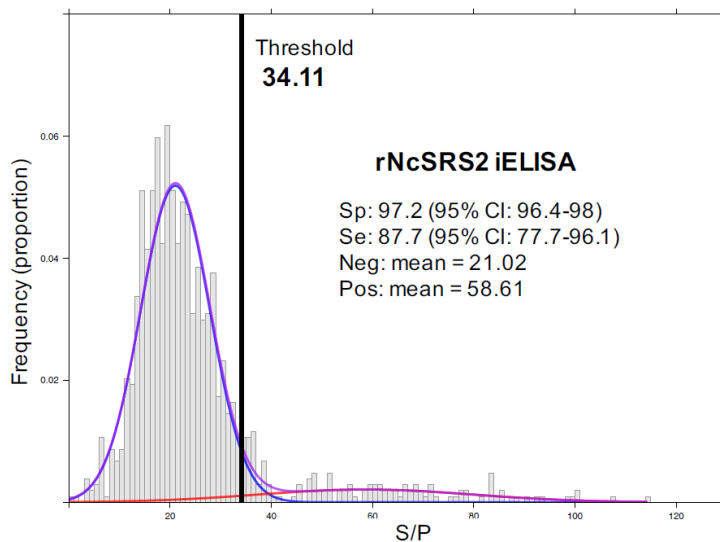
Optimum threshold values (defined as the highest total sensitivity and specificity of each test) were calculated from the fitted distributions based on the posterior distributional parameters and estimated at S/P value of 17.66, 34.11, 38.37, 52.62, 25.17 and 29.40 for the IDVet, rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs respectively (Figure 3.3).

Figure 3.3 – Frequency distribution of observed S/P ratios (%) in the IDVet (A), rNcSRS2 (B), rNcGRA7 (C), rNcSAG4 (D), rNcBSR4 (E) and rNcSRS9 (F) iELISAs ($n=1,037$) with fitted distributions using median estimates of the mean and variance of the two distributions from the MCMC analysis (solid blue and red curves). The compound distribution derived from the two constituent normal distributions is added on top (purple curve). The vertical black solid line represents the best estimate threshold associated with the optimised maximum sum for specificity and sensitivity.

(A)

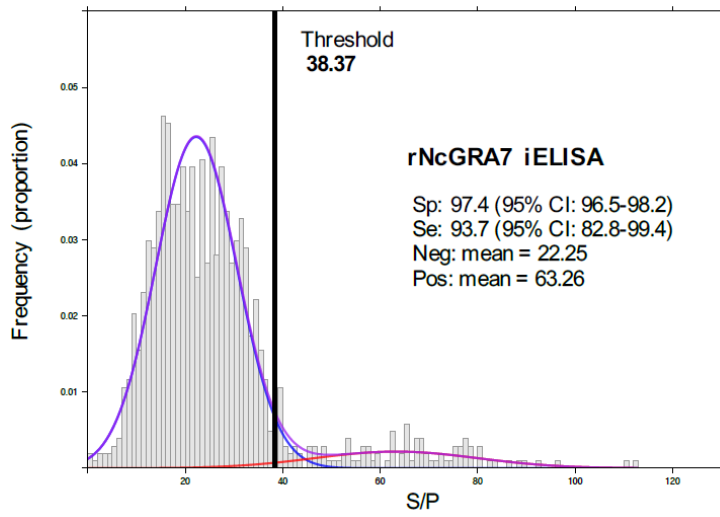


(B)

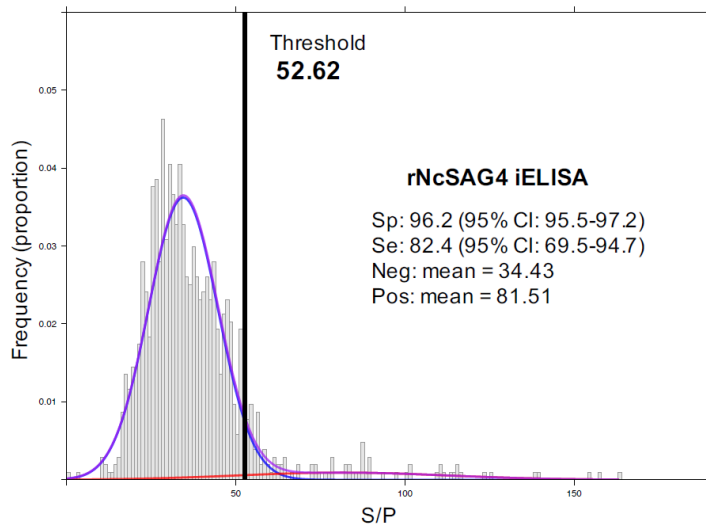


(continued)

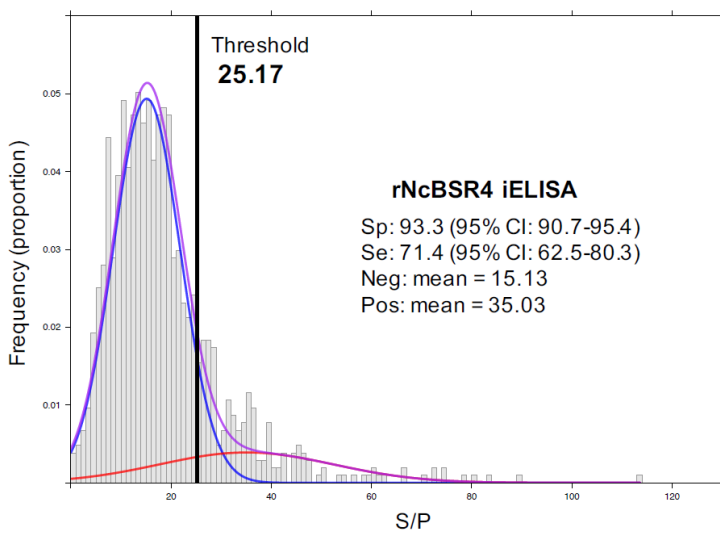
(C)



(D)

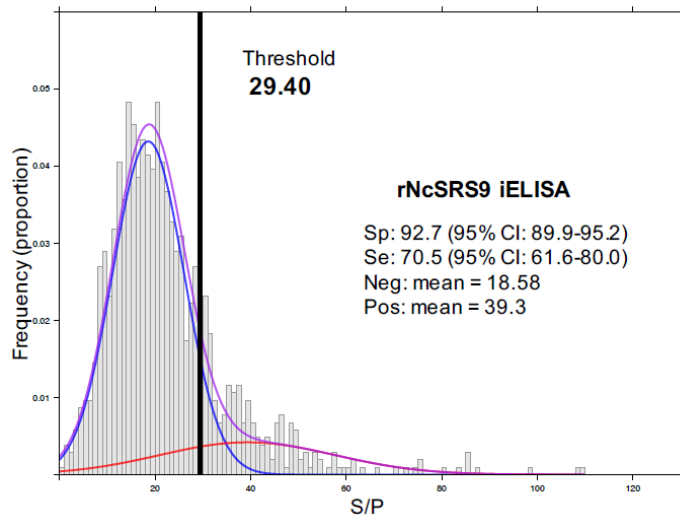


(E)



(continued)

(F)



From the estimated thresholds and the distributions fitted to the observed data, the model provided median sensitivity and specificity for each test analysed (Table 3.4). The IDVet iELISA showed the highest median sensitivity and specificity with estimated values of 99.6% (95% credible interval: 99.4-99.8) and 94.4% (89.1-98.4) respectively.

The experimental iELISAs based on the recombinant *N. caninum* immunodominant antigens rNcSRS2 and rNcGRA7 showed higher estimated sensitivity of 87.7% (77.7-96.1) and 93.7% (82.8-99.4) respectively compared to the recombinant bradyzoite-specific antigen-based iELISAs rNcSAG4, rNcBSR4 and NcSRS9 that had median sensitivity of 82.4% (69.5-94.7), 71.4% (62.5-80.3) and 70.5% (61.6-80.0) respectively.

In general, the estimated median specificity of the recombinant antigen-based iELISAs used was high and >90% in all tests: 97.2% (96.4-98.0), 97.4% (96.5-98.2), 96.2% (95.5-97.2) 93.3% (90.7-95.4), 92.7% (89.9-95.2) in rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs respectively (Table 3.4).

Table 3.4 – Median estimates representing the mean of the negative and positive distributions, optimum thresholds and median sensitivity (Se) and specificity (Sp) with 95% credible intervals.

Test	Negative median estimate (S/P)	Positive median estimate (S/P)	Threshold (S/P)	Se (%) (95% credible interval)	Sp (%) (95% credible interval)
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	7.57	77.06	17.66	94.4 (89.1-98.4)	99.6 (99.4-99.8)
rNcSRS2 iELISA	21.02	58.61	34.11	87.7 (77.7-96.1)	97.2 (96.4-98.0)
rNcGRA7 iELISA	22.25	63.26	38.37	93.7 (82.8-99.4)	97.4 (96.5-98.2)
rNcSAG4 iELISA	34.43	81.51	52.62	82.4 (69.5-94.7)	96.2 (95.5-97.2)
rNcBSR4 iELISA	15.13	35.03	25.17	71.4 (62.5-80.3)	93.3 (90.7-95.4)
rNcSRS9 iELISA	18.58	39.30	29.40	70.5 (61.6-80.0)	92.7 (89.9-95.2)

All test plasma samples were scored as antibody positive or negative with each test based on the estimated thresholds (Table 3.5). Similar proportions of antibody-positive cattle were obtained using the tests based on *N. caninum* recombinant antigens: 8.9% (95% CI: 7.3-10.8%), 9.2% (7.5-11.1) and 8.7% (7.1-10.5) for the IDVet, rNcSRS2 and rNcGRA7 iELISA respectively. Within the evaluation of the bradyzoite-specific antibody responses, 4.7% (3.6-6.2), 8.4% (6.8-10.2) and 7.0% (5.6-8.8) of the animals tested were classified as antibody-positive using the rNcSAG4, rNcBSR4 and rNcSRS9 iELISAs respectively (Table 3.5).

Table 3.5 – Number of plasma samples classified as antibody-positive and antibody-negative based on the estimated optimum thresholds for the 6 iELISAs used ($n=1,037$) and proportion of animals classified as antibody-positive (%). CI: confidence interval.

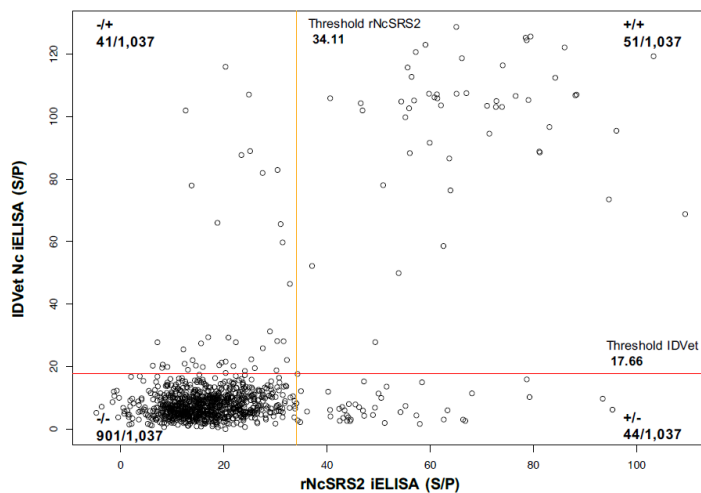
Test	Positive	Negative	Proportion (%) (95% CI)
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	92	945	8.9 (7.3-10.8)
rNcSRS2 iELISA	95	942	9.2 (7.5-11.1)
rNcGRA7 iELISA	90	947	8.7 (7.1-10.5)
rNcSAG4 iELISA	49	988	4.7 (3.6-6.2)
rNcBSR4 iELISA	87	952	8.4 (6.8-10.2)
rNcSRS9 iELISA	73	964	7.0 (5.6-8.8)

3.3.3 Test agreement

Test pairs were graphically visualised in scatterplots in which the ELISA results (expressed in S/P percentages), obtained with two tests, were plotted against each other. Each dot in the scatter plot represents one individual animal and its position depends on the results obtained with the two tests considered. The preliminary visual analysis of the ELISA data dispersion suggested that, in general, a limited number of animals tested seropositive with both tests considered regardless of the test pair compared (Figure 3.4 and 3.5).

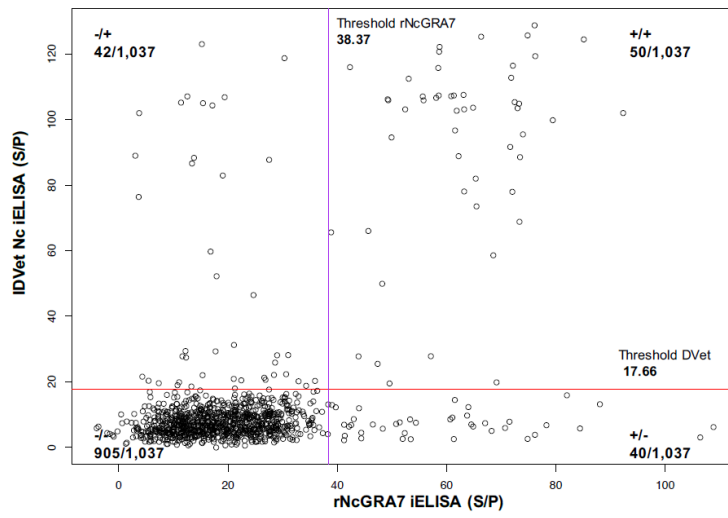
Figure 3.4 – Scatter plots of test pairs based on *N. caninum* tachyzoites lysate and immunodominant recombinant antigens considered to be markers of acute infection. (A) IDVet and rNcSRS2 iELISAs, (B) IDVet and rNcGRA7 iELISAs, (C) rNcSRS2 and rNcGRA7 iELISAs. The threshold S/P value for each test is indicated by a red, orange or purple line for the IDVet, the rNcSRS2 and the rNcGRA7 iELISAs respectively.

(A)



(continued)

(B)



(C)

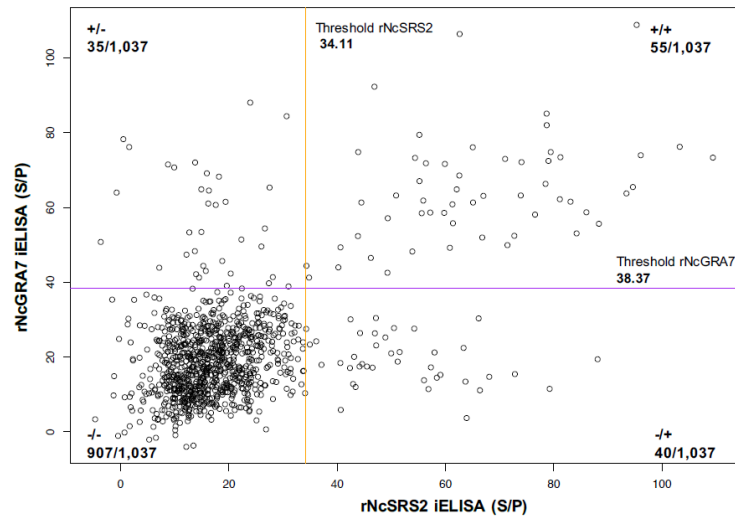
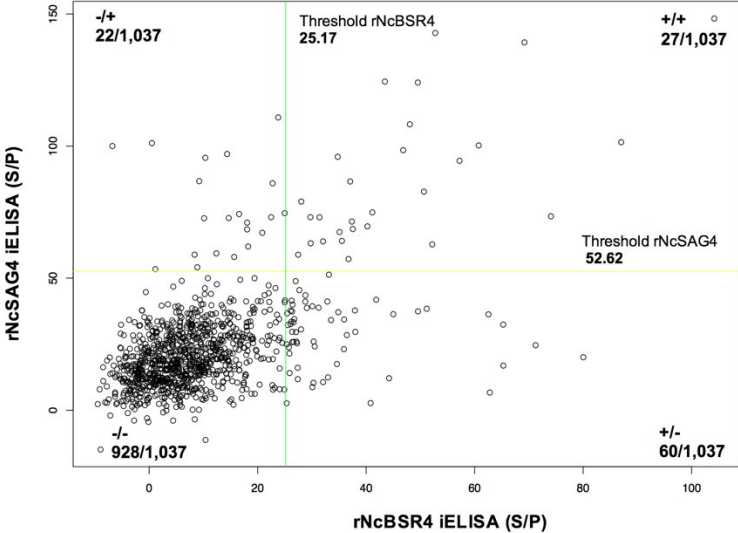
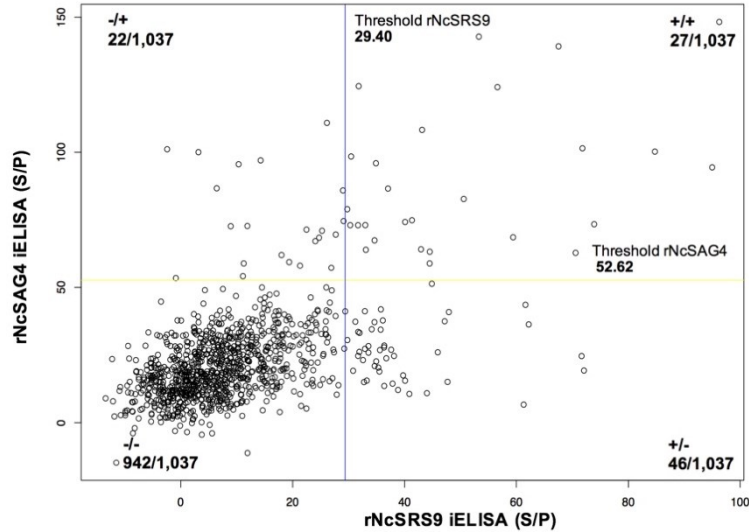


Figure 3.5 – Scatter plots of tests pairs based on *N. caninum* bradyzoite-specific antigens. rNcSAG4 and rNcBSR4 iELISAs, (B) rNcSAG4 and rNcSRS9 iELISAs, (C) rNcBSR4 and rNcSRS9 iELISAs. The threshold S/P value for each test is indicated by a yellow, green or blue line for the rNcSAG4, the rNcBSR4 and the rNcSRS9 iELISAs respectively.

(A)

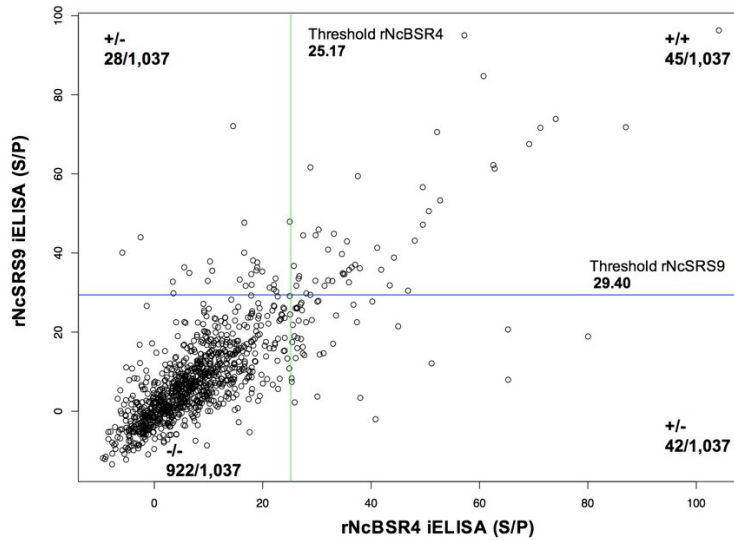


(B)



(continued)

(C)



The agreement between each combination of test pair, expressed as Kappa statistic (κ) with confidence intervals, is shown in Table 3.6. Moderate agreement ($\kappa = 0.41-0.60$) was observed between tests using *N. caninum* immunodominant antigens (IDVet, rNcSRS2 and rNcGRA7 iELISAs) or between tests using bradyzoite-specific antigens (rNcSAG4, rNcBSR4 and rNcSRS9). In contrast, tests using immunodominant antigens considered to be markers of acute infection showed only slight agreement ($\kappa = 0.01-0.20$) with tests using bradyzoite-specific antigens (Table 3.6).

Table 3.6 – Test agreement (κ) in different combinations of iELISA pairs with 95% confidence intervals. κ values indicative of moderate agreement between iELISAs targeting antibodies against *N. caninum* immunodominant antigens considered to be markers of acute infection are shaded in yellow whereas values indicating moderate agreement between pairs of iELISA based on bradyzoite antigens are shaded in green. In grey, κ values which are indicative of slight agreement between iELISAs based on antigens highly expressed by the tachyzoite stage and iELISAs which employ bradyzoite-specific antigens.

iELISA	rNcSRS2	rNcGRA7	rNcSAG4	rNcBSR4	rNcSRS9
IDVet	0.50 (0.41-0.59)	0.51 (0.42-0.60)	0.09 (0.01-0.18)	0.066 (-0.0089-0.14)	0.079 (0.0015-0.16)
rNcSRS2		0.55 (0.47-0.64)	0.21 (0.11-0.31)	0.086 (0.0084-0.16)	0.088 (0.0091-0.17)
rNcGRA7			0.19 (0.09-0.29)	0.14 (0.057-0.23)	0.095 (0.014-0.18)
rNcSAG4				0.42 (0.31-0.52)	0.47 (0.36-0.58)
rNcBSR4					0.58 (0.49-0.67)

Collectively, the outcome of the six iELISAs used consisted of 249 (24.0%, 95%CI: 21.5-26.7) animals with a positive result in at least one test and 788 (76.0%, 73.3-78.5) cattle which tested negative with all tests (Table 3.7).

Table 3.7 – Tested dairy cattle ($n=1,037$) with positive test results by number of assays with which they were classified as positive.

6 iELISAs outcome	No. Cows	No. positive tests	No. Cows
Positive (positivity with ≥ 1 test)	249	Positive with 6 tests	2
		Positive with 5 tests	6
		Positive with 4 tests	13
		Positive with 3 tests	59
		Positive with 2 tests	46
		Positive with 1 tests	123
Negative (negativity with all tests)	788		

Only 2 plasma samples tested antibody-positive with all six assays whereas 123 positive samples (50.6%, 44.4-56.7) were classified as such based on the positive outcome obtained with only one test of the six examined.

As a result of the reduced agreement between tests based on antigens considered to be markers of acute infection which are highly expressed by the tachyzoite stage and test-based bradyzoite-specific antigens, only 43 (35.0%, 27.1-43.7) of the 126 plasma samples which tested positive with more than one test had a positive result with at least one tachyzoite and one bradyzoite antigen-based iELISA at the same time (Table 3.8).

Table 3.8 – Number of animals with merged test results for the panel of the iELISAs based on antigens highly expressed by tachyzoite stage (T) and the panel of bradyzoite antigens-based iELISAs (B). T+/B+: cows which tested positive with at least one tachyzoite (T+)/bradyzoite (B+) antigens-based iELISA, T-/B-: cows which tested negative with all three tachyzoite (T-)/bradyzoite (B-) antigens-based iELISAs). Blue: positives with at least one tachyzoite and one bradyzoite iELISA. Yellow/green: positives with at least one tachyzoite (yellow)/bradyzoite (green) iELISA.

	T+ (No. Cows)	T- (No. Cows)	
B+ (No. Cows)	43	88	131
B- (No. Cows)	118	788	
	161		

Combinations of test results on the three iELISAs based on antigens highly expressed by the tachyzoite stage, that are considered as markers of acute infection, and the three tests based on bradyzoite-specific antigens, are detailed in Table 3.9.

Table 3.9 – Number of tested dairy cows ($n=1,037$) with specified combinations of test results on (A) one iELISA based on *N. caninum* tachyzoites lysate and two iELISAs based on recombinant *N. caninum* antigens considered to be markers of acute infection and (B) three iELISAs based on bradyzoite-specific antigens.

(A)

IDVet	rNcSRS2 iELISA	rNcGRA7 iELISA	No. Cows
Pos	Pos	Pos	41
Pos	Pos	Neg	10
Pos	Neg	Neg	32
Pos	Neg	Pos	9
Neg	Pos	Pos	14
Neg	Neg	Pos	26
Neg	Pos	Neg	30
Neg	Neg	Neg	875
			1037

(B)

rNcSAG4 iELISA	rNcBSR4 iELISA	rNcSRS9 iELISA	No. Cows
Pos	Pos	Pos	27
Pos	Pos	Neg	2
Pos	Neg	Neg	17
Pos	Neg	Pos	3
Neg	Pos	Pos	22
Neg	Neg	Pos	25
Neg	Pos	Neg	34
Neg	Neg	Neg	907
			1037

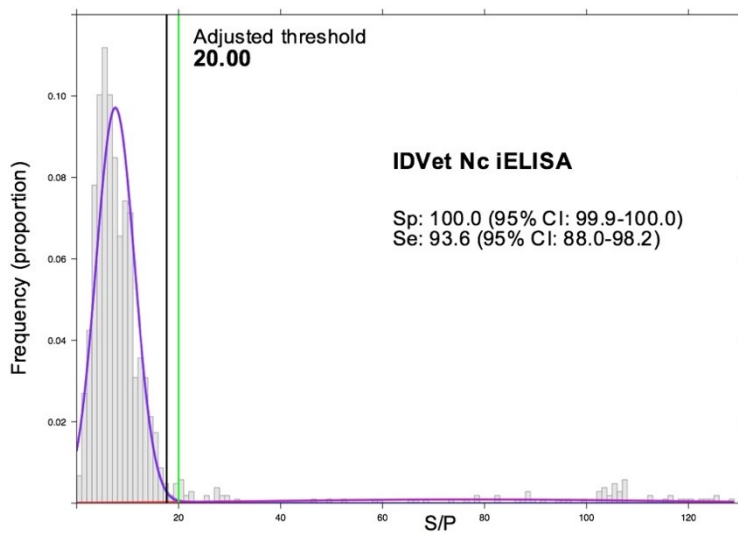
3.3.4 Threshold adjustment

In order to compare the six iELISAs, for which optimal cut-offs were calculated using the bimodal normal distribution model, a set of antibody-positive reference samples was generated. The threshold of each iELISA was adjusted so that all plasma samples with S/P values within the fitted distribution of negative samples were arbitrarily considered as negatives. This also resulted in the classification of previous positive samples (i.e. characterised by S/P values within the interval in which the fitted distribution of the negative samples overlapped the fitted distribution of the positive ones) as negatives. Such classification ensured that no negative samples were classified as positives. In this way, the specificity of each iELISA was maximised at the expense of the sensitivity (Figure 3.6). However, this arbitrary method was exclusively instrumental to the identification of reference plasma samples which were likely to be truly *N. caninum* antibody positive.

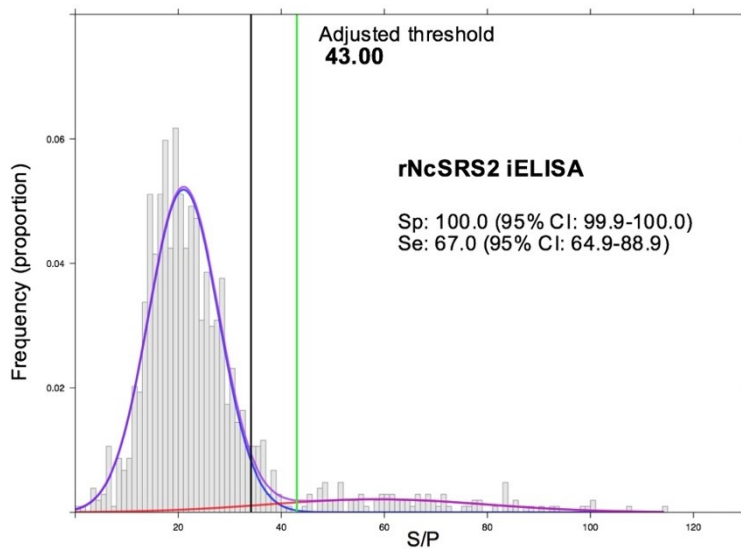
All plasma samples which tested antibody positive with at least one iELISA, based on the adjusted thresholds, were considered as truly positives. These samples were 173 whereas the samples which tested negative with all six iELISAs (considered as truly negatives) were 864. This panel of positive and negative samples was used as a reference against which the Se of each iELISA was reassessed (Table 3.10). The reassessment was carried out using the iELISA outcome based on the cut-off S/P values previously calculated using the bimodal normal distribution model (Table 3.4).

Figure 3.6 – Frequency distribution of observed S/P ratios (%) in the IDVet (A), rNcSRS2 (B), rNcGRA7 (C), rNcSAG4 (D), rNcBSR4 (E) and rNcSRS9 (F) iELISAs ($n= 1,037$) with fitted distributions using median estimates of the mean and variance of the two distributions from the MCMC analysis (solid blue and red curves). The compound distribution derived from the two constituent normal distributions is added on top (purple curve). The vertical black solid line represents the best estimate threshold associated with the optimised maximum sum for specificity and sensitivity whereas the vertical green line represents the threshold adjusted to maximise for specificity and eliminate false positive results.

(A)

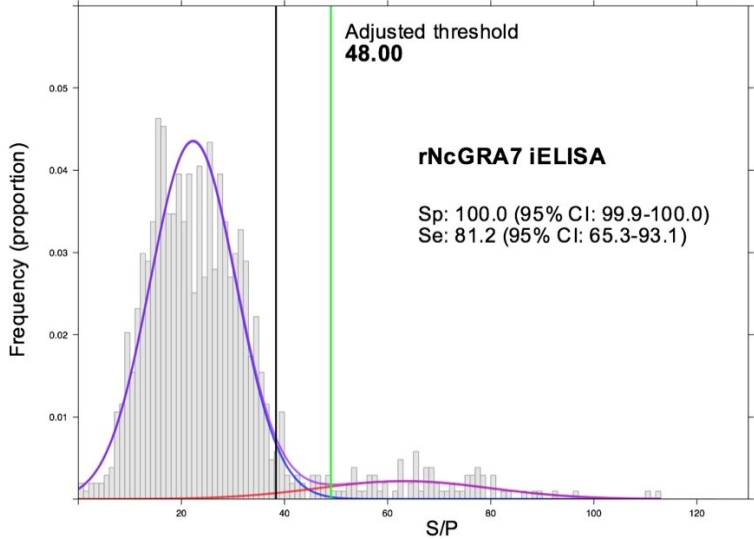


(B)

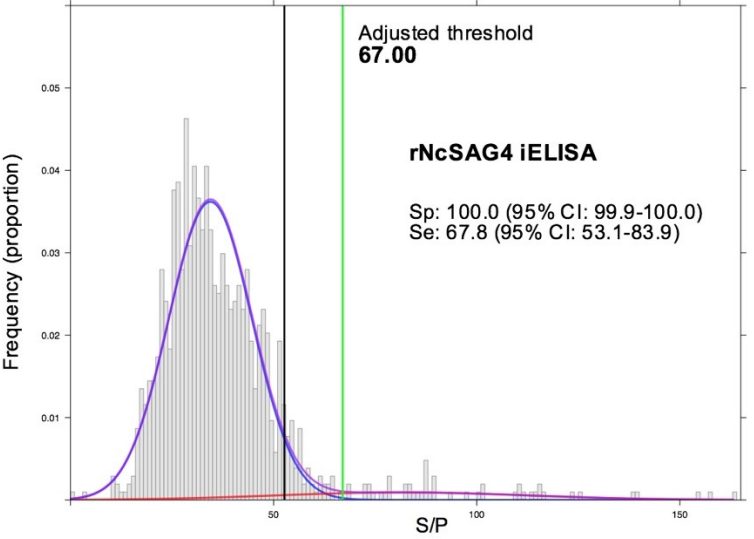


(continued)

(C)

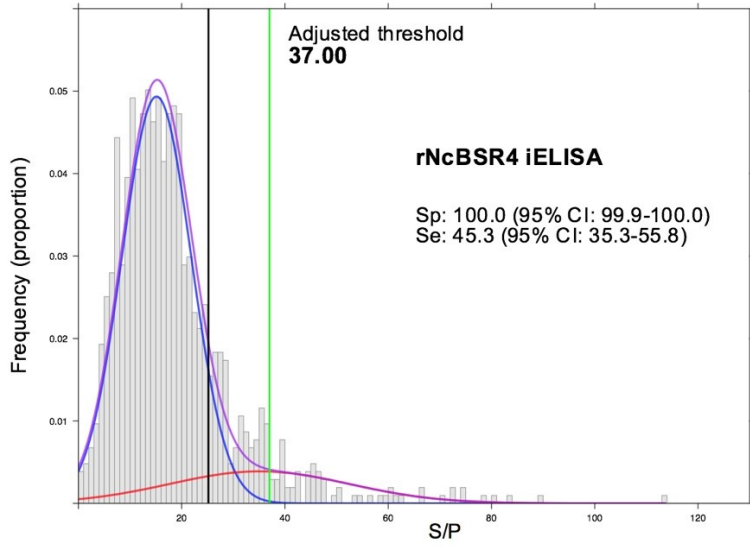


(D)



(continued)

(E)



(F)

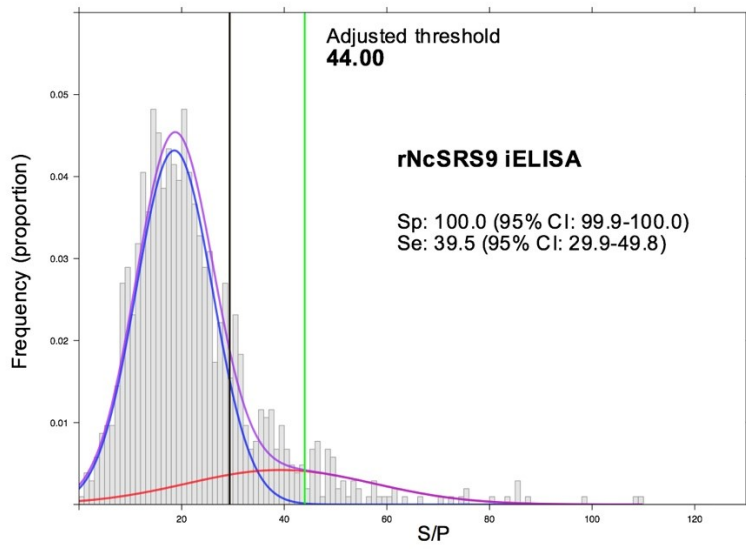


Table 3.10 – Specificity (Sp) and sensitivity (Se) of the six iELISAs calculated using a panel of reference plasma samples generated by adjusting the threshold of each test so that all S/P values falling within the fitted negative distribution were scored as negatives.

Test	Positive samples	Negative samples	False positive	False negative	Se*(%) (95%CI)	Sp*(%) (95%CI)
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	87	859	5	86	50.3 (42.6-58.0)	99.4 (98.6-99.8)
rNcSRS2 iELISA	85	854	10	88	49.13 (41.5-56.8)	98.8 (97.8-99.4)
rNcGRA7 iELISA	82	856	8	91	47.40 (39.7-55.1)	99.1 (98.2-99.6)
rNcSAG4 iELISA	40	855	9	133	21.4 (17.06-30.13)	99.0 (98.0-99.6)
rNcBSR4 iELISA	50	827	37	123	28.9 (22.3-36.3)	95.7 (94.1-97.0)
rNcSRS9 iELISA	44	835	29	129	25.4 (19.1-32.6)	96.7 (95.2-97.7)
Reference test	173¹	864²	0	0	100.0 (97.8-100.0)	100.0 (99.6-100.0)

¹ Reference positive: plasma samples which were classified as positive with at least one test.

² Reference negative: plasma samples which tested negative with all 6 tests.

* Based on the panel of reference samples generated.

The overall diagnostic performances obtained using multiple *N. caninum* iELISAs in parallel were also evaluated. The combination of two assays based on tachyzoite antigens enabled to improve the Se with an exiguous loss in Sp; the commercial test (IDVet) associated to either the rNcSRS2 or the rNcGRA7 iELISAs showed a Sp of 97.8% (95%CI: 96.6-98.7). The combination with rNcSRS2 provided a Se of 67.9% (60.1-74.5) whereas the in parallel use with rNcGRA7 gave a Se of 65.9% (58.3-72.3) (Table 3.11).

A serological diagnostic tool using all three iELISAs targeting antibody responses against the tachyzoite stage of *N. caninum* (subsequently denominated test A) showed increased Se (81.5%; 74.9-86.9) and acceptable Sp (95.6%, 94.0-96.9). In contrast, a serological diagnostic protocol using all tests based on bradyzoite-specific antigens showed low Se (43.3%, 35.8-51.1) as well as lower Sp compared to the use of the tachyzoite antigens-based iELISAs.

Within the tests based on either whole *N. caninum* tachyzoite lysate or recombinant immunodominant antigens considered to be markers of acute infection, the commercial IDVet iELISA associated with either the rNcSRS2 or the rNcGRA7 provided higher Se and Sp compared to the use in parallel of the two experimental tests based on recombinant proteins. Furthermore, the combination of the commercial

assay with either rNcSRS2 or rNcGRA7 and one recombinant bradyzoite antigen-based assay (rNcSAG4, rNcBSR4 and rNcSRS9) enabled to increase the Se. Nevertheless, this level of Se was similar to the Se obtained by using the IDVet, the rNcSRS2 and the rNcGRA7 iELISAs simultaneously.

Finally, a diagnostic tool based on all proposed iELISAs employing antigens highly expressed by the tachyzoite stage in association with a single bradyzoite antigen-based iELISA showed good Se and Sp (>90%). The Se and Sp of rNcSAG4 iELISA combined with the IDVet, rNcSRS2 and rNcGRA7 (test B) were 91.3% (86.1-95.1) and 96.6% (95.2-97.7) respectively. Notably, the rNcBSR4 iELISA had the greater impact on the improvement of the overall Se (94.8%, 90.3-97.6) (test C) (Table 3.11).

Table 3.11 – Diagnostic specificity (Sp) and sensitivity (Se) of selected combinations of iELISAs. Tested dairy cattle ($n=1,037$) with test results classified as positive, negative, falsely positive and negative based on the set of reference plasma samples generated by adjusting the S/P value cut-offs for each test.

Combination of iELISAs	Positive samples	Negative samples	False positives	False negatives	Se* (95%CI)	Sp* (95%CI)
IDVet + rNcSRS2	117	845	19	56	67.6 (60.1-74.5)	97.8 (95.6-98.7)
IDVet + rNcGRA7	114	845	19	59	65.9 (58.3-72.3)	97.8 (96.6-98.7)
rNcSRS2 + rNcGRA7	106	836	28	67	61.2 (53.6-72.3)	96.8 (95.3-97.8)
Test A: IDVet + rNcSRS2 + rNcGRA7	141	843	21	32	81.5 (74.9-86.9)	97.6 (96.3-98.5)
rNcSAG4 + rNcBSR4	68	835	29	105	39.3 (31.09-47.0)	96.6 (95.2-97.7)
rNcSAG4 + rNcSRS9	59	827	37	114	34.1 (27.1-41.7)	95.7 (94.1-97.0)
rNcBSR4 + rNcSRS9	61	811	53	112	35.2 (28.2-42.8)	93.9 (92.1-95.4)
rNcSAG4 + rNcBSR4 + rNcSRS9	75	805	59	98	43.3 (35.8-51.1)	93.2 (91.3-94.8)
IDVet + rNcSRS2 + rNcSAG4	141	840	24	32	81.5 (74.9-87.0)	97.2 (95.9-98.2)
IDVet + rNcGRA7 + rNcSAG4	141	842	22	32	81.5 (74.9-87.0)	97.4 (96.2-98.4)
IDVet + rNcSRS2 + rNcBSR4	150	816	48	23	86.7 (80.7-91.4)	94.4 (92.7-95.8)
IDVet + rNcGRA7 + rNcBSR4	146	818	46	27	84.4 (78.1-89.5)	94.6 (93.0-96.1)
IDVet + rNcSRS2 + rNcSRS9	144	819	45	29	83.2 (76.9-88.5)	94.8 (93.1-96.20)
IDVet + rNcGRA7 + rNcSRS9	142	821	43	31	82.1 (75.5-87.5)	95.1 (93.3-96.4)
Test B: IDVet + rNcSRS2 + rNcGRA7 + rNcSAG4	158	834	30	15	91.3 (86.1-95.1)	96.5 (95.1-97.6)
Test C: IDVet + rNcSRS2 + rNcGRA7 + rNcBSR4	164	809	55	9	94.8 (90.3-97.6)	93.6 (91.8-95.2)
IDVet + rNcSRS2 + rNcGRA7 + rNcSRS9	160	813	51	13	92.5 (87.5-95.9)	94.1 (92.3-95.6)
rNcSRS2 + rNcGRA7 + rNcSAG4 + rNcBSR4	140	828	24	33	80.9 (74.3-86.5)	97.2 (97.2-98.2)
rNcSRS2 + rNcGRA7 + rNcSAG4 + rNcSRS9	134	812	52	39	77.5 (70.5-83.4)	94.0 (92.2-95.5)
rNcSRS2 + rNcGRA7 + rNcBSR4 + rNcSRS9	134	815	49	39	77.5 (70.5-83.4)	94.3 (92.6-95.8)
Reference test	173¹	864²	0	0	100.0 (97.8-100.0)	100.0 (99.6-100.0)

¹ Reference positive: plasma samples which were classified as positive with at least one test based on the adjusted cut-off. ²Reference negative: plasma samples which tested negative with all 6 tests.

* Based on the panel of reference samples generated.

3.3.5 Seroprevalence of *N. caninum* in British dairy cattle

In order to enable easier comparison with previous estimates, the overall seroprevalence in dairy cattle is reported according to the results obtained with the commercial IDScreen® *Neospora caninum* indirect ELISA (IDVet). Similarly, to the tests employed in previous studies (Brickell *et al.*, 2010, Woodbine *et al.*, 2008), this test utilises native *N. caninum* tachyzoite extract as antigen preparation.

Based on the cut-off of the IDVet test (S/P=17.66) obtained using the bi-modal normal mixture model previously described, the serological results were dichotomised into positives and negatives: 92 cows out of 1,037 were classified as seropositive, giving an apparent seroprevalence of 8.9% (95% CI: 7.3-10.8).

In order to obtain the true prevalence (TP), the apparent prevalence (AP) was corrected according to the Rogan-Gladen estimator that takes into account sensitivity and specificity of the test used ($TP = (AP + Sp - 1) / (Se + Sp - 1)$) (Rogan and Gladen 1978) thus providing adjustment for misclassification by the diagnostic test. Considering that the Sp and Se calculated using the bi-modal normal mixture model were 99.6% and 94.4% respectively, the true overall prevalence would have been estimated at 9.0% (7.3-11.0). The apparent overall seroprevalence estimated using all 3 tests based on tachyzoite antigens in parallel (test A) or a combination of all iELISAs based on antigens which were considered to be markers of acute infection with the rNcSAG4 (test B) or the rNcBSR4 (test C) iELISA was estimated at 15.6% (13.5-18.0), 18.1% (15.9-20.6) and 21.1% (18.7-23.7) respectively (Table 3.12).

Table 3.12 – *N. caninum* seroprevalence in British dairy cattle ($n=1,037$) estimated using a commercial antibody ELISA (IDVet) and three serological tools combining all iELISAs based on antigens highly expressed by the tachyzoite stage (test A) or all iELISAs considered in test A associated with one experimental iELISA based on one bradyzoite-specific antigen (test B and C).

Test	Positive samples	Negative samples	Seroprevalence (%) (95%CI)
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	92	945	8.9 (7.3-10.8)
Test A (IDVet + rNcSRS2 + rNcGRA7)	162	875	15.6 (13.5-18.0)
Test B (IDVet + rNcSRS2 + rNcGRA7 + rNcSAG4)	188	849	18.1 (15.9-20.6)
Test C (IDVet + rNcSRS2 + rNcGRA7 + rNcBSR4)	219	818	21.1 (18.7-23.7)

In comparison to a serological tool based on 3 different iELISAs based on antigens expressed by the tachyzoite stage (test A) or these 3 tests associated with either the rNcSAG4 (test B) or the rNcBSR4 (test C) bradyzoite antigen-based ELISAs, the seroprevalence detected with the commercial test (IDVet) was lower by 6.7%, 9.2% and 12.2% respectively.

Considering the outcome of the IDVet iELISA, 41 herds out of 61 had at least one cow which was classified as seropositive (67.2%, 54.0-78.7). In these herds, the number of seropositive cows ranged from 1 to 12 (mean 2.2, median 2) corresponding to a mean within-cluster seroprevalence of 13.0% (10.1-16.0). Most clusters of 17 animals had only one (18/41, 43.9%, 29.9-59.0) or two (13/41; 31.6; 19.6-47.0) seropositive cows. In one cluster, 12 cows out of 17 were classified as antibody positive (Figure 3.7).

Based on the outcome of test A, B and C, the between-herd seroprevalence was 93.4% (57/61, 84.3-97.4), 95.1% (58/61, 86.5-98.3) and 98.3 (60/61, 91.3-99.7). The frequency distribution of the number of animals classified as seropositive in each herd with the four serological tools considered is shown in Figure 3.7.

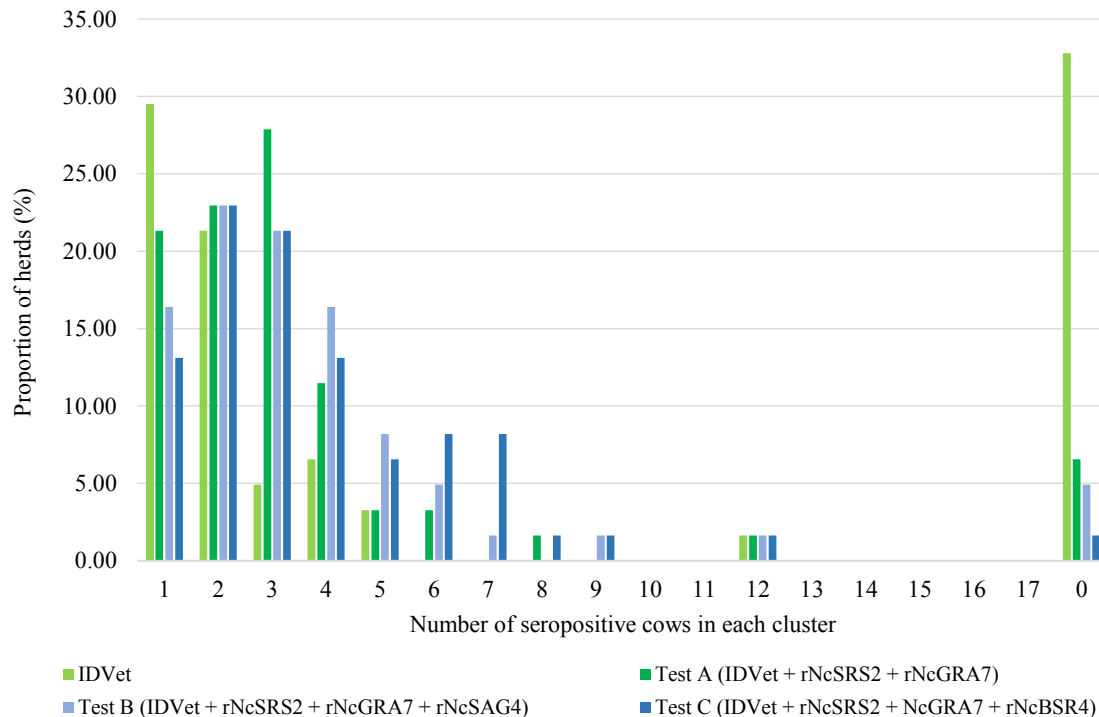


Figure 3.7 – Frequency distribution of the number of *N. caninum* seropositive cows in the sampled herds ($n=61$) as estimated with the commercial test (IDVet) and different combinations of the IDVet iELISA associated with experimental assays based on recombinant tachyzoite immunodominant and bradyzoite-specific antigens (Test A, B and C).

The observed individual seroprevalence in relation to the region of origin of the samples is shown in Table 3.13. The highest seroprevalences based on the outcome of the commercial tests were observed in the North East, Yorkshire and the Humber (17.6%, 11.5-26.2) and North West England (11.8%, 7.7-17.0) compared to the other British macro-regions. Using test A and B the highest seroprevalences were found in cattle from the North East, Yorkshire and the Humber (test A: 27.4%, 19.7-36.8; test B: 30.4%, 22.3-39.9) and the Midlands (test A and B: 22.3%, 14.8-32.8). If the outcome of test C is considered, the North East of England, Yorkshire and the Humber and Wales had the highest seroprevalence: 29.4% (95%CI: 21.4-38.9 and 16.8-46.2). However, only 2 Welsh herds were included in the sample (Table 3.13).

Table 3.13 – Observed individual seroprevalence of *N. caninum* in relation to the region of origin, in British dairy cattle ($n=1,037$) estimated with the commercial test (IDVet) and the serological tools denominated Test A, B and C.

Region	Cattle tested (n)	IDVet (95% CI)	Test A ¹ (95% CI)	Test B ² (95% CI)	Test C ² (95% CI)
Scotland	238	6.7 (16) (4.2-10.6)	12.6 (30) (3.8-10.6)	14.7 (35) (10.7-19.7)	18.1 (43) (13.7-23.4)
North East England Yorkshire and the Humber	102	17.6 (18) (11.5-26.2)	27.4 (28) (19.7-36.8)	30.4 (31) (22.3-39.9)	29.4 (30) (21.4-38.9)
North West England	204	13.2 (27) (9.3-18.6)	19.1 (39) (14.3-25.1)	20.6 (42) (15.6-26.7)	25.0 (51) (19.6-31.4)
Midlands	85	8.2 (7) (4.0-16.0)	22.3 (19) (14.8-32.3)	22.3 (19) (14.8-32.3)	25.9 (22) (17.8-36.1)
Wales	34	2.9 (1) (1.5-14.9)	14.7 (5) (6.4-30.1)	20.6 (6) (10.3-36.8)	29.4 (10) (16.8-46.2)
East of England	51	3.9 (2) (1.1-13.2)	13.7 (7) (6.8-25.7)	17.6 (9) (9.6-30.2)	25.5 (13) (15.5-38.9)
South East England	51	7.8 (4) (3.1-18.5)	11.8 (6) (5.5-23.4)	11.8 (6) (5.5-23.4)	15.7 (8) (11.6-27.5)
South West England	272	6.2 (17) (3.9-9.7)	10.3 (28) (7.2-14.5)	14.3 (39) (10.7-19.0)	15.4 (42) 11.6-20.2
All	1,037	8.9 (7.3-10.8)	15.6 (13.5-18.0)	18.1 (15.9-20.6)	21.1 (18.7-23.7)

¹ IDVet, rNcSRS2 and rNcGRA7 used in parallel.

² IDVet, rNcSRS2, rNcGRA7 and rNcSAG4 used in parallel.

³ IDVet, rNcSRS2, rNcGRA7 and rNcBSR4 used in parallel.

In general, the observed seroprevalence increased with the lactation number. However, a higher seroprevalence was observed amongst the heifers compared to animals during their first lactation, irrespective of the serological test or combination of tests considered (Table 3.14).

Table 3.14 – Observed individual seroprevalence of *N. caninum*, in relation to parity, in British dairy cattle ($n=952$) sampled in 56 herds assessed with the commercial test (IDVet) or the serological tools denominated Test A, B and C.

Parity	Cattle tested (n)	IDVet (95% CI)	Test A ¹ (95% CI)	Test B ² (95% CI)	Test C ² (95% CI)
0 (heifers)	88	9.1 (8) (4.7-16.9)	18.2 (16) (11.5-27.5)	18.2 (16) (11.5-27.5)	19.3 (17) (12.4-28.8)
1	144	5.6 (8) (2.8-10.6)	11.8 (17) (7.5-18.1)	15.3 (22) (10.3-22.0)	17.4 (25) (12.0-24.4)
2	251	6.8 (17) (4.3 -10.6)	14.7 (37) (10.9-19.7)	17.5 (44) (13.3-22.7)	21.5 (54) (16.8-27.0)
3	205	8.3 (17) (5.2-12.8)	16.6 (34) (12.1-22.3)	18.0 (37) (13.4-23.9)	21.5 (44) (16.4-27.6)
4	137	9.5 (13) (5.6-15.6)	17.5 (24) (12.1-24.7)	20.4 (28) (14.5-27.9)	22.6 (31) (16.4-30.3)
> 4	127	13.4 (17) (8.5-20.4)	14.2 (18) (9.2-21.3)	19.7 (25) (13.7-27.4)	22.8 (29) (16.4-30.1)
All	952	8.4 (80) (6.8-10.3)	15.3 (146) (13.7-17.8)	18.1 (172) (15.7-20.6)	21.0 (200) (18.5-23.7)

¹ IDVet, rNcSRS2 and rNcGRA7 used in parallel.

² IDVet, rNcSRS2, rNcGRA7 and rNcSAG4 used in parallel.

³ IDVet, rNcSRS2, rNcGRA7 and rNcBSR4 used in parallel.

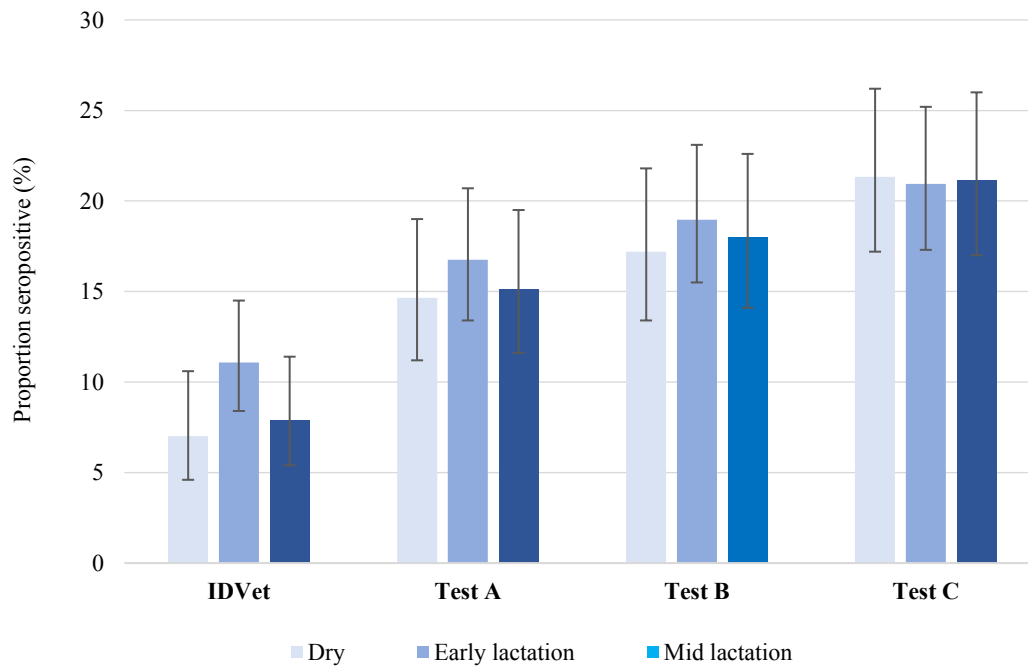
3.3.5.1 Seroprevalence in different production groups of dairy cattle

Based on the outcome of the commercial iELISA (IDVet), the observed proportions of serologically positive cattle during the dry period, in early lactation and mid-lactation were 7.0% (22/314, 95% CI: 4.7-10.4), 11.1% (45/406, 8.4-14.5) and 7.9% (25/317, 5.4-11.4) respectively (Figure 3.8). The seroprevalence observed in cattle in early lactation was higher compared to the seroprevalence in cows during the dry period; however, analysed within the GLMM, described previously, this difference was only just statistically significant ($p=0.049$) (OR=1.75, 95% CI: 1.000 – 3.066). In addition, there was no statistically significant difference in the seroprevalence between mid-lactation and the dry period ($p=0.657$) (OR=1.15, 0.617 – 2.147) (Table 3.21).

Similar, although lower, seroprevalence variations between productive/reproductive groups were obtained using the combination of iELISAs based on whole tachyzoite lysate (IDVet) and recombinant antigens considered to be markers of acute infection (rNcSRS2 and rNcGRA7) (test A) and the test associated with the bradyzoite-specific rNcSAG4 (test B). Minimal variation in the seroprevalence of the 3 groups were observed when all iELISAs based on antigens highly expressed by the tachyzoite stage were used in combination with the bradyzoite-specific rNcBSR4 (test C). Using test C, the observed seroprevalence in dry, mid-lactating and early lactating cows were 21.3% (67/314, 17.3-26.2), 20.9% (85/406, 17.3-25.2) and 21.1% (67/317, 17.0-26.0) respectively (Figure 3.8).

The differences in seroprevalence observed among the productive/reproductive groups, using test A, B and C, were not statistically significant irrespective of the test combination considered (Table 3.21).

Marginal variations in point estimates and p values in multivariate GLMMs, suggested that there was no evidence that the lactation number represented a confounder in the present study. Interaction between lactation number and productive/reproductive group was also excluded. Therefore, the results here presented refer to the GLMM fitted considering herd of origin as a random effect and productive group (Early lactation, Mid-lactation and Dry) as fixed effect (Table 3.15).



Test A: IDVet, rNcSRS2 and rNcGRA7 used in parallel.
Test B: IDVet, rNcSRS2, rNcGRA7 and rNcSAG4 used in parallel.
Test C: IDVet, rNcSRS2, rNcGRA7 and rNcBSR4 used in parallel.

Figure 3.8 – Seroprevalence of *N. caninum* in cattle in early lactation ($n=406$), mid-lactation ($n=317$) and during the dry period ($n=314$) estimated with the commercial test (IDVet) and the serological tools denominated Test A, B and C. Error bars indicate exact binomial 95% confidence intervals.

Table 3.15 – Seroprevalence of *N. caninum* by IDVet iELISA or combinations of tests carried out in parallel denominated test A, B or C in cattle in early lactation ($n=406$), mid-lactation ($n=317$) and during the dry period ($n=314$) with GLMM outcome. OR: Odds Ratio.

Test	Productive /reproductive group	Seroprevalence (%) (95%CI)	Significance (p) (significance level $p<0.05$)	OR (95%CI)
IDScreen® <i>Neospora caninum</i> iELISA (IDVet)	Dry	7.0 (4.7-10.4)	intercept	-
	Early	11.1 (8.4-14.5)	0.049	1.75 (1.00-3.06)
	Mid	7.9 (5.4-11.4)	0.657	1.15 (0.61-2.14)
Test A (IDVet + rNcSRS2 + rNcGRA7)	Dry	14.6 (11.2-19.0)	intercept	-
	Early	16.7 (13.4-20.7)	0.430	1.18 (0.78-1.79)
	Mid	15.1 (11.6-19.5)	0.842	1.05 (0.66-1.64)
Test B (IDVet + rNcSRS2 + rNcGRA7 + rNcSAG4)	Dry	17.3 (13.4-21.8)	intercept	-
	Early	19.0 (15.5-23.1)	0.473	1.15 (0.78-1.72)
	Mid	18.0 (14.1-22.6)	0.834	1.04 (0.69-1.59)
Test C (IDVet + rNcSRS2 + rNcGRA7 + rNcBSR4)	Dry	21.3 (17.2-26.2)	intercept	-
	Early	20.9 (17.2-26.2)	0.922	0.98 (0.68-1.42)
	Mid	21.1 (17.0-25.0)	0.934	0.98 (0.64-1.46)

3.4 Discussion

Updated information on the seroprevalence of *N. caninum* in a cattle population is a prerequisite for shaping appropriate control programmes for bovine neosporosis. Furthermore, baseline seroprevalence data are needed to enable the reliable interpretation of serological results obtained from aborting dams within the investigation of abortion outbreaks (Davison *et al.*, 1999c, McAllister 2016).

One of the primary objectives of this study was to determine the seroprevalence of *N. caninum* in British dairy cattle.

Five experimental antibody ELISAs based on recombinant antigens of *N. caninum* (rNcSRS2, rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9) were used alongside a commercial test which was considered as a representative of the assays commonly used within serological surveys. Indeed, a previous study reported excellent agreement between the IDScreen® *Neospora caninum* indirect ELISA (IDVet) and most commercially available serological tests for bovine neosporosis which are based on the same principle and similar antigenic preparations (Alvarez-Garcia *et al.*, 2013).

A commercial iELISA based on native NcSRS2 surface antigen (Bio K 192, Monoscreen Ab ELISA, Bio-X Diagnostics, Rochefort, Belgium) (Ghalmi *et al.*, 2009) was available at the time of the study, the in-house rNcSRS2 iELISA was used for costs reasons.

All six tests were used to test a sample of the British adult dairy cattle population for *N. caninum*-specific antibodies in plasma.

Given the unavailability of a true gold standard test for bovine neosporosis (Ortega-Mora *et al.*, 2007) and a sufficient number of reference plasma samples, traditional methods to obtain a cut-off value with estimates of the characteristics of each test were inadequate (Jacobson 1998). For this reason, a bi-modal normal distribution model was used. The model fitted the data and a threshold value for each test was obtained.

Estimation of the test characteristics from the model showed that Se and Sp of the tests based on antigens highly expressed by the tachyzoite stage and considered as markers of acute infection were superior compared to those of the tests based on bradyzoite-specific antigens. Amongst the tests using antigens expressed by the tachyzoite stage, the commercial iELISA based on total extract of sonicated tachyzoites (IDVet) was

characterised by the highest estimated median Se (94.4%) and Sp (99.6%) followed by the experimental assay based on the recombinant NcGRA7 antigen (rNcGRA7) (Se: 93.7%, Sp:97.4%). Although characterised by adequate median Sp (97.2%), the rNcSRS2 iELISA showed lower median Se compared to the other two tests (87.7%). Within the group of tests based on bradyzoite antigens, the rNcSAG4 iELISA was characterised by the best median Sp (96.2%) and Se (82.4%) compared to the rNcBSR4 and the NcSRS9 iELISAs which showed lower median Se of 71.4% and 70.5% respectively.

Interestingly, the lower Se of the rNcSAG4 iELISA compared to the rNcGRA7 iELISA is consistent with previous evaluations of these two tests (Aguado-Martinez *et al.*, 2008). Aguado-Martinez *et al.*, (2008) reported 96.0% and 89.0% Se for the rNcGRA7 and rNcSAG4 iELISA respectively; however, a direct comparison of test characteristics is not possible as the calculation of the optimal cut-offs, Se and Sp was carried out using methods based on a gold standard reference test and a panel of reference sera, implemented with TG-ROC analysis (Aguado-Martinez *et al.*, 2008). Moderate, but not high, agreement was found amongst tests based on antigens highly expressed by the tachyzoites, either native (IDVet) or recombinant (rNcSRS2 and rNcGRA7) and amongst bradyzoite-specific antigen-based iELISAs (rNcSAG4, rNcBSR4 and rNcSRS9).

High levels of agreement between diagnostic ELISAs may reflect the similarity of test principle, technical aspects (e.g. plasma and conjugate dilutions) and antigen preparation (Alvarez-Garcia *et al.*, 2013). Tests based on different antigenic preparations may show lower levels of agreement because of the detection of antibodies directed against different epitopes of the parasite, expressed at different lifecycle stages of the parasite (Dubey *et al.*, 1997, Jenkins *et al.*, 2000). Therefore, the moderate agreement observed amongst tests targeting antibodies responses against antigens which are expressed by the same stage of *N. caninum* may be due to the different antigenic preparation and technical characteristics of each iELISA protocol. Notably, the only slight agreement observed when each iELISA employing antigens which are highly expressed by the tachyzoite stage was compared with each bradyzoite antigen-based iELISA may indicate that the two types of antigenic preparation are recognised by different antibody responses against *N. caninum*. These two aspects do

not necessarily overlap. Only a relatively small proportion (35.0%) of animals which tested positive with more than one test showed detectable antibodies against both tachyzoite and bradyzoite antigens (Tables 3.7-3.8).

Most *N. caninum* antibody positive cows tested positive with only one test or showed detectable plasma antibodies exclusively against antigens predominantly expressed by the tachyzoite, but not bradyzoite-specific antigens, and vice versa. In practical terms, this may imply that the exclusive use of serological assays based on tachyzoite immunodominant antigens may result in false negative results. Such false negative results are not taken into account by the Se of the test used because cattle infected with *N. caninum* may have detectable antibody titres against antigens expressed by the bradyzoite stage but not against immunodominant antigens of the tachyzoite stage.

The combined analysis of test results obtained with both tests based on antigens highly expressed by the tachyzoite stage, which are considered to be markers of acute infection, and bradyzoite-specific antigens, which have potential for identification of persistently infected animals, can provide information on the stage of infection (i.e. primary infection, recrudescence, reinfection, or persistent infection) as described in a previous study in which the rNcGRA7 and the rNcSAG4 iELISAs were used in parallel (Aguado-Martinez *et al.*, 2008).

In the present study, the background information available (e.g. history of *N. caninum* abortions at the herd and individual level) was not sufficient to support any evaluation of the stage of infection. Nonetheless, it might be possible to speculate that, out of the total number of animals with at least one positive iELISA result, cattle with at least one tachyzoite and one bradyzoite positive result (43/249; 17.3%, 95%CI: 13.1-22.4%) were likely to be animals in which a recent acute infection became quiescent or cows in which reactivation or reinfection occurred. Cattle which tested positive with iELISAs using antigens highly expressed by *N. caninum* tachyzoites only (118/249; 47.4%, 41.3-53.6%) experienced a primary exposure to the parasite and animals which tested positive with bradyzoite antigens-based tests only (88/249; 35.3%, 29.7-41.5%) harboured the quiescent stage of *N. caninum* within a persistent infection.

Since the infection dynamics with regards to the level of exposure of bradyzoite antigens during the latent stage of *N. caninum* are currently unclear. The evaluation of tests targeting antibody responses elicited by the bradyzoite stage of the parasite is

difficult. Tissue cyst rupture or reactivation would be required for these antigens to be exposed to the host immune system. Further specific longitudinal studies are therefore required to assess how these mechanisms influence the presence of circulating stage-specific antibodies in persistently infected cows. Although in one study the rNcSAG4 iELISA was applied to the analysis of sera from persistently infected cows sampled longitudinally (Aguado-Martinez *et al.*, 2008), additional work using an increased number of animals and a wider panel of iELISAs based on bradyzoite-specific antigens may help to clarify the presence and temporal dynamics of antibody titres against the quiescent stage of *N. caninum*.

Combined results using more than one test showed that the Se of the serological diagnosis can be enhanced using multiple tests in parallel. However, there are practical and economic limitations to the number of tests that can be used routinely.

Although the IDVet, rNcSRS2 and rNcGRA7 iELISAs used in combination and associated to one of the bradyzoite antigen-based assays were collectively characterised by Se and Sp > 90%, the simultaneous use of four tests would require careful cost-benefit analysis due to the increased costs. Alternatively, the development of one antibody ELISAs using a mixture of recombinant antigens to coat a single well may be explored as a strategy for the reduction of the costs involved.

The use of three tests would provide a Se of 81.5% regardless of whether the commercial test is used alongside both rNcSRS2 and rNcGRA7 iELISAs or one of these two tests associated with the rNcSAG4 iELISA. These test combinations would provide comparable Sp (Table 3.11).

Interestingly, the combination of all three tests based on antigens highly expressed by the tachyzoite stage with either the rNcBSR4 or the rNcSRS9 iELISA determined a slightly higher increase in Se compared to the use of the rNcSAG4 iELISA associated with these three tests. However, the univariate analysis and distribution of the results obtained with the rNcBSR4 and rNcSRS9 iELISAs highlighted generally poor performances which suggested the need for further optimisation (Figure 3.3 – E-F).

3.4.1 Preliminary estimation of false negative results obtained with iELISAs based exclusively on *N. caninum* tachyzoite antigens

A set of reference samples was generated by adjusting the cut-off of each iELISA to identify animals that were truly positive by scoring likely false positive results as negatives and by combining the test results obtained with all tests. This enabled the comparison of individual tests and combinations of tests against a panel of reference positive and negative samples based on all iELISAs evaluated.

The use of all three assays based on antigens which are considered to be markers of acute infection (test A) enabled the identification of 81.5% (141/173; 95%CI: 75.0-86.6) of the samples deemed as *N. caninum* antibody positive thus suggesting that approximately 18.5% false negative results could be attributed to the fact that assays based on bradyzoite-specific antigens were not included (Table 3.11). This would provide an approximate estimation of the number of animals that would not be correctly identified because, although they have bradyzoite-specific circulating antibodies, the levels of tachyzoite-specific antibodies are not detectable. Such estimate was supported by the increase in seroprevalence when one test based on bradyzoite antigens is used in parallel with all three tests based on tachyzoite antigens. The overall seroprevalence assessed with test B and C increased by 2.5% (from 15.6 to 18.1%) and 5.5% (from 15.6 to 21.1%) respectively corresponding to an increment of 14.0% and 26.0% (Table 3.12).

The reliable estimation of the proportion of animals which may truly show antibodies against the quiescent stage of *N. caninum* was hampered by the poor Se displayed by the rNcSAG4 iELISA and the inadequate Se and Sp of the rNcBSR4 and rNcSRS9 iELISAs observed within the univariate analysis of these tests (Figure 3.3). This suggested that further optimisation and validation of these tests are required.

Importantly, also tests based on immunodominant antigens predominantly expressed by the tachyzoite stage showed limited sensitivity when used singularly. If the outcome of the commercial test (IDVet) is considered alone, the proportion of animals classified as seronegative but in which levels of anti-*N. caninum* antibodies were detectable with at least one of the other tests would be as high as 46.8% (81/173, 39.5-54.2) (Table 3.10). This indicated that a large proportion of infected animals may be misclassified

as seronegative if a single commercial assay based on antigens considered to be markers of acute infection (immunodominant tachyzoite antigens) was used.

In the absence of assays which would provide improved performances, combining the results of different tests carried out in parallel should be considered as a strategy to improve the sensitivity of the serological diagnosis of bovine neosporosis.

According to the analysis performed, 3 iELISAs based on immunodominant antigens expressed by the tachyzoite stage of *N. caninum* associated with 1 iELISA using a recombinant bradyzoite-specific antigen, either rNcSAG4 (test B) or rNcBSR4 (test C), would provide satisfactory Se and Sp levels (Table 3.11). Although not perfect, these serological tools may represent a valuable alternative to overcome the poor performances in terms of sensitivity observed when assays based exclusively on immunodominant tachyzoites antigens, used individually or in combination (test A), are employed for determining the *N. caninum* serological status in cattle.

3.4.2 Seroprevalence of *N. caninum* in British dairy herds

The proportion of *N. caninum* serologically positive animals is influenced by several factors and varies depending on the country, region and herd considered. In addition, the serological techniques and specific tests used as well as the cut-offs applied contribute significantly to the variations detected in different studies. The seroepidemiological data presented in this cross-sectional study are based on the outcome of a commercially available ELISA (IDVet) applied on a large sample of British adult dairy cattle. Likewise, most of the ELISAs used in previous seroprevalence studies, the commercial test employed used native *N. caninum* tachyzoite antigens for the detection of specific IgGs. Recalculation of the cut-off value of this test was carried out using the bimodal normal mixture model. Importantly, using this cut-off the test showed the highest estimated median Se and Sp compared to the other tests assessed.

The overall *N. caninum* seroprevalence was estimated at 9.0%. This is similar to previous estimates of 6% (6%; 95% CI: 4-8) (Davison *et al.*, 1999c) in normally calving dairy cattle in England and Wales and 12.9% in dairy and suckler bovines, longitudinally tested at yearly intervals for four years, in south-west England (Woodbine *et al.*, 2008). In the latter study, 87.1% (86.1-87.6) of the cows always

tested negative, 8.1% (7.6-8.6) always tested positive whereas 4.8% (4.5%-5.2%) had different test results at different time points (Woodbine *et al.*, 2008). A higher seroprevalence (17.1%) was reported in a cross-sectional serological survey conducted on a sample of English dairy cattle; however, only herds with history of *N. caninum* abortions were selected in this study (Davison *et al.*, 1999a).

If the cut-off suggested by the manufacturer of the IDVet iELISA (i.e. S/P=50, with samples in which $40 < S/P < 50$ classified as doubtful) had been chosen, 60 cows out of 1,037 would have been classified as seropositive thus giving a seroprevalence of 5.8% (4.5-7.4). Two animals would have had a doubtful test result. Sp and Se of the IDVet iELISA on the basis of the manufacturer's cut-off were calculated by Alvarez-García *et al.* (2013) who used gold standard criterion defined pre-test information (i.e. reference positive and negative serum samples classified based on previous epidemiological, clinical and serological data). Sp and Se were 98.3% (96.3-100) and 98.9% (97.6-100) respectively (Alvarez-Garcia *et al.*, 2013). Based on this information, the true estimated prevalence, corrected as previously described using the Rogan-Gladen estimator would have been 5.9 %.

Interestingly, using the rNcSRS2 and rNcGRA7 iELISAs similar seroprevalence estimates were obtained: 9.2% and 8.7% respectively which corrected based on the Rogan-Gladen estimator were 7.5% and 6.7% respectively.

Collectively all tests based on antigens predominantly expressed by the tachyzoite stage (test A) classified as *N. caninum*-antibody positive 162 animals generating an apparent overall seroprevalence of 15.5% (13.5-18.0). Including the outcome of one assay based on bradyzoite antigens the estimated proportion of seropositive cows was 18.1% (188/1,037; 15.9-20.6) with the rNcSAG4 (test B) and 21.1% (219/1,037; 18.7-23.7) with the rNcBSR4 iELISA (test C). These combinations of assays were selected because they were characterised by good Se with minimal decrease in Sp (test B) or by the highest Se (test C) as calculated using the reference panel of samples generated (Table 3.11).

The percentage of herds in which serological evidence of *N. caninum* was found was high (67.2%, 54.0-78.7% with IDVet) or very high (>90% with test A, B or C) thus confirming that the protozoan parasite is relatively common in dairy herds across Great Britain. In other European countries, the between-herd seroprevalence of *N. caninum*

at the national level is highly variable. A supranational large-scale serological study reported serological positivity for the parasite in 16%, 49%, 63% and 76% of dairy herds sampled in Sweden, Germany, Spain and the Netherlands respectively (Bartels *et al.*, 2006a) using ELISAs that were based on whole tachyzoite extracts as antigen preparations.

Bayesian MCMC methods represent an alternative strategy for the estimation of the seroprevalence. These techniques have been previously successfully applied to estimate the prevalence of animal and human infectious diseases using serological data (Opsteegh *et al.*, 2010; Xia *et al.*, 2013). In contrast to frequentist approaches, Bayesian MCMC simulations incorporate prior information about the parameters of each test, which are used to reach an overall best estimate of the prevalence (Basanez *et al.*, 2004). In the present study, such methods were employed for the calculation of the optimal cut-off values and the analysis of the characteristics (Sp and Se) of the serological assays used in order to overcome the lack of a gold standard test for bovine neosporosis.

3.4.2.1 Seroprevalence in different production groups of dairy cattle

The serological screening of cattle in mid to late pregnancy has been traditionally advised as it is thought to give the best chances of revealing infected animals (Dannatt 1997). In clinical practice, it is often recommended to serologically test cows during drying off, which is typically done 8 weeks before the predicted calving date, according to the general husbandry of dairy cattle (Figure 3.8). In addition, the current voluntary control scheme for bovine neosporosis proposed by the Cattle Health Certification Standards (CHeCS) (i.e. the self-regulatory body for cattle health schemes in the UK and Ireland) recommends testing pregnant animals between 12 and 4 weeks pre-calving (CHeCS 2016). This sampling window is supported by the rise of *N. caninum*-specific antibody titres which was observed after the fifth month of gestation in naturally infected cows within a number of longitudinal studies (Andrianarivo *et al.*, 2005, Cardoso *et al.*, 2009, Dannatt 1997, Guy *et al.*, 2001). In addition, the occurrence of most neosporosis-induced abortions during the 5th-6th month of gestation (Dubey 2003) led to the biological assumption that in non-aborting dams the reactivation of a quiescent infection would be likely to occur at this stage or

later. Reactivation would result in increased exposure of the parasite to the host immune system thus triggering an increase in detectable antibody levels. According to these observations, a higher seroprevalence amongst animals in late gestation would be expected.

In the present study, the GLMM analysis of the serological data, which was performed to consider the farm of origin as a random effect, showed limited differences in the seroprevalence of three groups of animals sampled during three productive phases (early lactation, mid-lactation and dry period). These three productive stages reflect, with some approximation, three reproductive stages: non-gestation/uterine involution, early pregnancy and late gestation respectively (Figure 3.9).

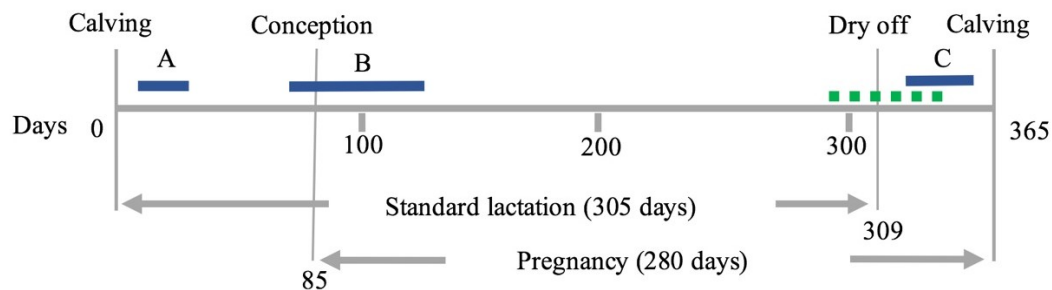


Figure 3.9 – Standard dairy cows’ productive cycle with blood sampling time intervals (blue lines): (A) early lactation (B) mid-lactation and (C) dry period. The green dashed line indicates the 12 to 4 weeks pre-calving sampling window historically recommended by the main UK provider of cattle health schemes for bovine neosporosis.

In contrast to the hypothesis of a higher seroprevalence amongst cattle in late pregnancy, the observed proportion of *N. caninum* antibody-positive animals in the group of dry cows was (7.0%) similar and not significantly different to seroprevalence of cows in mid-lactation (7.9%) ($p=0.657$). Careful interpretation of the higher seroprevalence observed in early lactating cows (11.1%) is required as the difference from the seroprevalence during the dry period was only just statistically significant ($p=0.049$). Furthermore, on average, there were only approximately 30 days between the sampling time of early lactating (days in milk: mean \pm SD 18.7 ± 7.2) and dry cows (days prior to the expected calving date: 11.4 ± 9.6).

Additionally, equivalent GLMM analysis carried out based on the results obtained with different combination of assays used in parallel (Test A, B and C) showed that

there were no statistically significant differences between the seroprevalence observed in dry cows compared to the seroprevalence in early or mid-lactation.

Overall, these findings indicate the lack of strong evidence to suggest that carrying out serological investigations for bovine neosporosis during a specific productive/reproductive stage of the three investigated would increase the chances of detecting infected animals.

Due to the analysis of residual samples originally collected for a different purpose, the direct assessment of the actual validity of the recommended sampling window (12 to 4 weeks pre-calving) was difficult since most of the dry cows were sampled less than 4 weeks pre-calving (days prior to the expected calving date: mean=11.4, median=7.0, min=1, max=82).

In dairy cattle, the concentration of total IgGs in peripheral blood steadily decreases from the 8th week (Herr *et al.*, 2011) or the 5th-4th week (Detilleux *et al.*, 1995, Franklin *et al.*, 2005) ante-partum to parturition. Such pregnancy-associated phenomenon is considered physiological and most likely caused by the translocation of immunoglobulins to the mammary tissue for colostrum production (Herr *et al.*, 2011). The decline of total IgGs towards term might have had an impact on the proportion of dry cows which showed *N. caninum*-specific plasma IgGs above the cut-off of the iELISA considered. Indeed, the vast majority of the animals in this group were close to calving hence to the stage in which the concentration of total IgGs is at its minimum. In summary, the cross-sectional data produced did not support the exclusive serological examination of cows during the dry period; however, further investigations are necessary.

Longitudinal studies of a large number of cattle are required to investigate the dynamics of antibody titres during the different productive/reproductive stages and their impact on the serological diagnosis of bovine neosporosis under field conditions. This would help to identify the optimal time frame, if any, during which serological testing ought to be performed.

3.5 Conclusions

Tests based on tachyzoite antigens alone showed generally better performances than tests based on bradyzoite antigens. However, the use of tests based on bradyzoite antigens should be considered because of the evidence that a proportion of animals may display antibodies against antigens expressed by the quiescent stage of the parasite but not against the rapidly multiplying tachyzoite stage. These findings confirmed the concerns related to the potential lack of sensitivity of serological assays based exclusively on tachyzoite antigens. Such shortcoming may be overcome by carrying out multiple tests based on both *N. caninum* tachyzoite and bradyzoite antigens in parallel. This strategy would be particularly beneficial in those clinical scenarios in which high sensitivity is required, for example, to identify *N. caninum* infected cattle to cull or exclude from breeding replacement stock, or to screen prospective replacement cattle.

The cross-sectional seroprevalence study showed that *N. caninum* is widespread amongst British dairy herds. In addition, the data collected did not support that testing animals in late pregnancy would increase the likelihood of detecting cattle infected with *N. caninum*. However, further longitudinal studies are required to corroborate this finding.

Chapter 4: Application of microsatellite markers to determine the genetic diversity of *N. caninum*

4.1 Introduction

Neospora caninum is a ubiquitous apicomplexan that has been detected in cattle in every country in which specific investigations were carried out. The parasite is expected to be widespread anywhere where definitive canid hosts and intermediate hosts are present (Goodswen *et al.*, 2013). Since the first isolations of the parasite from infected dog pups (NC-1 isolate) (Dubey *et al.*, 1988b) and aborted bovine fetuses (BPA-1 and BPA-2 isolates) (Conrad *et al.*, 1993a) in the United States, many *N. caninum* isolates have been characterised from different host species and geographical areas (Al-Qassab *et al.*, 2010b).

A number of studies showed that several genetic and biological characteristics are not strictly conserved within the *N. caninum* species and substantial differences exist between isolates. Variations in virulence and *in vitro* growth rate are well documented (Atkinson *et al.*, 1999, Pereira Garcia-Melo *et al.*, 2010, Quinn *et al.*, 2002b, Regidor-Cerrillo *et al.*, 2010, Schock *et al.*, 2001). In cattle, Nc-Liverpool, which was originally isolated in the UK from the brain of a congenitally infected dog (Barber *et al.*, 1995), is characterised by high virulence and was able to readily induce abortion in experimentally infected pregnant animals (Williams *et al.*, 2000). Likewise, NC-1 caused foetal death in intravenously challenged pregnant heifers; in contrast, no foetopathy was observed in experimental infections conducted with the Nc-Spain 1H isolate (Rojo-Montejo *et al.*, 2009a). In a non-pregnant BALB/c mouse model of *N. caninum* infection, 8 Spanish isolates showed biological differences in terms of temporal dissemination of the parasite, parasite burden and histopathological lesions in the brains and lungs of infected mice (Pereira Garcia-Melo *et al.*, 2010). A similar infection model showed that Nc-Liverpool infection results in severe clinical signs and histopathological lesions in the central nervous system whereas milder clinical and pathological findings are seen following infection with the Swedish isolate Nc-SweB1 (Atkinson *et al.*, 1999). Differences in pathogenicity of *N. caninum* isolates were also observed in pregnant BALB/c mouse models. In one study, three Spanish isolates (Nc-

Spain 4H, Nc-Spain 5H, Nc-Spain 7) and Nc-Liverpool were associated with the onset of clinical signs in the dams and a higher neonatal mortality rate compared with another six Spanish isolates which showed lower morbidity (Regidor-Cerrillo *et al.*, 2010). *In vitro*, the same panel of *N. caninum* isolates displayed significant differences in the invasion rate (i.e. median number of intracellular parasites at a specific time) and proliferation kinetics (i.e. number of tachyzoites at a given time point measured by quantitative PCR). Interestingly, increased invasion rate and proliferation kinetics *in vitro* were correlated with high morbidity in mice (Regidor-Cerrillo *et al.*, 2011). A degree of phenotypic and genotypic heterogeneity is expected for a parasite with few geographical barriers, a broad range of intermediate hosts and the capacity for sexual reproduction (Schock *et al.*, 2001).

The current knowledge of *N. caninum* sexual reproduction is scarce and mainly based on data extrapolated from the closely related apicomplexan *T. gondii*. Similar to *T. gondii* in the feline definitive host, *N. caninum* sexually reproduces in the intestinal cells of the canid host (Dubey *et al.*, 2007). Although enteroepithelial schizont-like stages of *N. caninum* were noted in one dog pup (Kul *et al.*, 2015), life cycle stages related to gametogony have not been demonstrated to date. Based on information obtained from *T. gondii*, sexual crosses between two different isolates simultaneously infecting the definitive host may occur (Grigg *et al.*, 2001). This may lead to recombination and reassortment of genetic material between the parental genomes of the isolates involved in the coinfection, thus resulting in new isolates with distinct genotypic and phenotypic features (Grigg *et al.*, 2001, Regidor-Cerrillo *et al.*, 2013). Furthermore, it has been postulated that the genetic material of *T. gondii* may undergo meiotic recombination during the brief diploid stages within the predominantly haploid life cycle of the parasite (Grigg and Sundar 2009). The actual occurrence of these mechanisms in *N. caninum* is yet to be verified; however, they would represent possible sources of genetic variability.

Discriminatory molecular methods are a key requirement for investigating the genetic heterogeneity of *N. caninum* and its implications in the epidemiology and pathogenesis of neosporosis (Beck *et al.*, 2009).

Preliminary studies used randomly amplified polymorphic DNA-PCR (RAPD-PCR) to assess the diversity amongst *N. caninum* isolates (Atkinson *et al.*, 2000, Schock *et*

al., 2000) and to differentiate *N. caninum* from *N. hughesi* (Spencer *et al.*, 2000). Based on short arbitrary primers that can bind different sites of the parasite genome, this technique provides limited detection of polymorphisms and is heavily reliant on the purity of the DNA template as well as on the amplification conditions (Al-Qassab *et al.*, 2010b).

The analysis of ribosomal DNA (18S subunit) has been used for phylogenetical studies of *Neospora* spp. and several related apicomplexans (Marsh *et al.*, 1995). At this level, sequence conservation amongst *N. caninum* isolates was found (Barber *et al.*, 1995, Marsh *et al.*, 1995, Stenlund *et al.*, 1997). This was attributed to the rare occurrence of nucleotide changes in the 18S sequences during *N. caninum* evolution from its closest ancestor (Al-Qassab *et al.*, 2010b, Marsh *et al.*, 1995).

Although minor variations of the internal transcribed spacer sequence (ITS1) have been documented amongst Brazilian (Nc-Bahia), European (Nc-Liverpool and Nc-SweB1) and North American (NC-1, Nc-Beef and Nc-Illinois) isolates, the polymorphism observed was considered insufficient for enabling differentiation at the isolate level (Gondim *et al.*, 2004a).

Other potentially polymorphic *loci* such as genes encoding the NcSAG1, NcSRS2 and NcSAG4 surface antigens (Fernandez-Garcia *et al.*, 2006, Marsh *et al.*, 1999), α -tubulin (Siverajah *et al.*, 2003), β -tubulin and HSP70 (McInnes *et al.*, 2006a) were also investigated revealing sequence conservation or extremely limited variations amongst isolates (Beck *et al.*, 2009). Finally, polymorphisms of the Nc5 repeat region of *N. caninum* genome were analysed (McInnes *et al.*, 2006a); however, only small variations between isolates were detected (Gondim *et al.*, 2004c, Wapenaar *et al.*, 2006).

Currently, the multilocus analysis of micro- and minisatellites is considered the gold standard for the genotyping of *N. caninum* (Al-Qassab *et al.*, 2010b, Donahoe *et al.*, 2015, Goodswen *et al.*, 2013). Micro- and minisatellites are tandemly repeated sequences of nucleotides which are widely distributed throughout the genomes of eukaryotic organisms (Tautz and Renz 1984). Microsatellites are short (2-6 nucleotides long) repetitive DNA motifs whereas minisatellites are characterised by longer repeated sequences (≥ 8 nucleotides) (Al-Qassab *et al.*, 2009). Within these *loci* the recurring addition or deletion of repeat units (e.g. due to DNA replication errors

such as, for example, slipped-strand mispairing) result in sequence length polymorphisms (Gemayel *et al.*, 2012); as a consequence, micro- and minisatellites are evolutionary relevant and represent useful DNA markers for the investigation of genetic diversity (Vieira *et al.*, 2016). Typically, the determination of the number of tandem repeats at multiple *loci* within the genome is carried out by PCR amplification followed by amplicon sizing using capillary electrophoresis (CE) or sequencing (Basso *et al.*, 2010). Microsatellite amplification by nested PCR showed higher sensitivity than conventional PCR (Basso *et al.*, 2009, Pedraza-Diaz *et al.*, 2009); therefore, it is generally preferred.

Several polymorphic *N. caninum* micro- and minisatellite *loci* have been identified to date. Distinct genetic profiles amongst 9 laboratory-maintained *N. caninum* isolates were found analysing 13 microsatellite markers (MS1A, MS1B, MS2, MS3, MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, MS21) (Regidor-Cerrillo *et al.*, 2006). The same panel of markers was subsequently used to characterise 9 Spanish isolates obtained from asymptomatic infected calves. Unique genetic patterns were observed between most isolates; however, those originating from the same herd showed identical or similar genetic profiles (Regidor-Cerrillo *et al.*, 2008).

The application of these markers to clinical samples (i.e. brains of aborted bovine foetuses) provided useful epidemiological information. Sub-clustering of isolates according to their geographical origin was described in one study (Pedraza-Diaz *et al.*, 2009). Additionally, shared microsatellite patterns, observed in aborted foetuses from individual abortion outbreaks, supported the hypothesis that the epidemic pattern of bovine neosporosis results from horizontal transmission from a point source of infection (Basso *et al.*, 2010).

Further investigations enabled the identification of additional polymorphic tandem repeats regions highlighting that also minisatellite *loci* show a significant degree of polymorphism that can be used for the study of the intraspecific genetic variation (Al-Qassab *et al.*, 2009). In this context, multiplex PCR amplification of micro- and minisatellite markers was proposed as a convenient tool for the differentiation of isolates and strains (Al-Qassab *et al.*, 2010a, Al-Qassab *et al.*, 2009).

Substantial genetic diversity amongst isolates has been observed showing that *N. caninum* exists as a diverse and genetically heterogeneous population (Al-Qassab *et*

al., 2009, Al-Qassab *et al.*, 2010b, Basso *et al.*, 2009, Beck *et al.*, 2009, Regidor-Cerrillo *et al.*, 2013, Rojo-Montejo *et al.*, 2009b).

The genotyping of *N. caninum* by multilocus micro- and minisatellites analysis has multiple applications. These include the investigation of abortion outbreaks, the identification and discrimination of vaccine and challenge isolates within live vaccine research and the study of population genetics (Basso *et al.*, 2010, Goodswen *et al.*, 2013, Regidor-Cerrillo *et al.*, 2013). In particular, the application of molecular typing tools to bovine abortion outbreaks may provide valuable information on the source of infection and the predominant route of transmission, thus helping to shape control strategies for bovine neosporosis at the herd level.

Suitable markers, characterised by adequate discriminatory power, are essential for the development of informative multilocus micro-/minisatellite tools.

The current study aims to:

1. Develop a panel of novel microsatellite *loci* to extend knowledge on polymorphic repetitive DNA sequences found in the *N. caninum* genome
2. Investigate the applicability of the expanded microsatellite markers panel to study genetic diversity of *N. caninum* in both laboratory-maintained and clinical samples.

4.2 Materials and methods

4.2.1 Laboratory-maintained *N. caninum* isolates

A total of 14 laboratory-maintained *N. caninum* isolates, which originated from either bovine ($n=10$) or canine ($n=4$) hosts from different geographical areas, were analysed in this study (Table 4.1). All isolates were obtained from cryopreserved tachyzoites stocks held at the Moredun Research Institute.

Tachyzoites of the Nc-LivB1, Nc-Spain2, Nc-JAP2 and Nc-JAP4 isolates were resuscitated from cryopreserved stocks and propagated in adherent and confluent monolayers of African Green Monkey Kidney (VERO) cells grown in 25 cm² tissue culture flasks (Thermo Fisher Scientific, Waltham, MA, USA). Cells and parasites were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with foetal bovine serum (4% v/v), penicillin G (50 U/ml) (Sigma-Aldrich, Munich, Germany) and streptomycin (50 µg/ml) (Sigma-Aldrich) at 37°C in a 5% CO₂ humidified incubator. Tachyzoites were harvested from the cell monolayer by disruption with a sterile cell scraper (Corning, NY, USA) and washed three times by centrifugation at 2,000×g for 5 minutes and resuspension in sterile PBS (pH 7.4). The parasites were counted using a Neubauer haemocytometer (Sigma-Aldrich). Pellets containing approximately 5×10^7 tachyzoites were prepared and stored at -80°C until DNA extraction.

DNA samples of the other isolates were extracted directly from cryopreserved $2-5 \times 10^7$ parasites aliquots stored in freezing medium (50% v/v heat inactivated foetal bovine serum, 40% v/v IMDM and 10% v/v dimethyl sulfoxide (DMSO)). Prior to DNA extraction, the content of each cryogenic tube (Nalgene™, General Long-Term Storage Cryogenic Tubes, Thermo Fisher Scientific, Waltham, MA, USA) was thawed slowly on ice, resuspended in 10 ml cold PBS and centrifuged at 2,000×g for 5 minutes in order to remove the freezing medium. The resulting parasite pellet was then stored at -80°C until genomic DNA preparation.

Table 4.1 – Laboratory-maintained *N. caninum* isolates used in this study: host, geographic origin and source.

Isolate	Host	Country of origin	Reference	Source
NC-1	Canine	USA	Dubey <i>et al.</i> , 1988	Congenitally infected dog pup (brain)
BPA1	Bovine	USA	Conrad <i>et al.</i> , 1993	Aborted foetus (brain)
JAP-2	Bovine	Japan	Yamane <i>et al.</i> , 1998	Congenitally infected calf (brain)
JAP-4	Bovine	Japan	Yamane <i>et al.</i> , 1998	Congenitally infected calf (brain)
Nc-Beef	Bovine	USA	McAllister <i>et al.</i> , 1998	Naturally infected calf
Nc-Drachten	Bovine	Netherlands	-	Naturally infected dog (oocysts)
Nc-Freiburg02	Canine	Germany	-	Naturally infected dog (oocysts)
Nc-Freiburg03	Canine	Germany	-	Naturally infected dog (oocysts)
Nc-GER1	Canine	Germany	Peters <i>et al.</i> , 2000	Congenitally infected dog pup (brain and spinal cord)
Nc-LivB1	Bovine	UK	Davison <i>et al.</i> , 1999	Stillborn calf (brain)
Nc-Poland	Bovine	Poland	Cabaj <i>et al.</i> , unpublished	Aborted foetus (brain)
Nc-Porto	Bovine	Portugal	Canada <i>et al.</i> , 2002	Aborted foetus (brain)
Nc-Spain2	Bovine	Spain	Regidor-Cerillo <i>et al.</i> , 2008	Naturally infected calf (brain)
Nc-SweB1	Bovine	Sweden	Stenlund <i>et al.</i> , 1997	Stillborn calf (brain)

4.2.2 Clinical samples

DNA samples extracted from brain, heart or placenta of 28 bovine aborted fetuses, in which *N. caninum* DNA was previously detected by ITS1 nested PCR (Katzner *et al.*, unpublished), were included in the present study. All abortions originated from 13 farms in the Dumfries and Galloway region in south-west Scotland and were collected between March 2008 and June 2011.

4.2.3 Genomic DNA extraction

4.2.3.1 Laboratory-maintained *N. caninum* isolates

Parasite pellets from both cultured and cryopreserved *N. caninum* tachyzoites (~2-5×10⁷ parasites) were resuspended in 100 µl PBS, pre-treated with proteinase K (50 mg/ml) (Sigma-Aldrich, Munich, Germany), mixed thoroughly and incubated at 56°C overnight. Genomic DNA was then extracted and purified using the DNeasy® Blood & Tissue Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Following the same protocol, DNA samples were extracted from uninfected VERO cells and subsequently used as negative controls.

Concentration and purity of the DNA samples were determined by spectrophotometry using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA purity was assessed using the absorbance ratio at 260 nm and 280 nm. DNA samples were diluted to a final concentration of 100 ng/μl with DNase/RNase free water (Sigma-Aldrich, Munich, Germany) and stored at -20°C until used in PCR.

4.2.3.2 Clinical samples

Foetal tissues (brain or heart) or placental cotyledons were previously homogenised using 2 ml Precellys® tubes containing ceramic beads and a Precellys® homogeniser (both Bertin Instruments, Montigny-le-Bretonneux, France) in which 1 g of tissue and 1 ml of Nuclei Lysis Solution (Promega, Southampton, UK) were added (Katzner and Bartley, unpublished).

DNA extraction was performed from tissue homogenate as outlined in Bartley *et al.* (2013b) with minor modifications. Briefly, 400 μl of homogenate was added to 900 μl of Nuclei Lysis Solution and incubated overnight at 55°C. Once cooled, 300 μl of Protein Precipitation Solution (Promega) was added. The mixture was mixed thoroughly, incubated on ice for at least 5 min and centrifuged at 13,000×g for 5 min. The resulting supernatant was then transferred to a 2 ml tube containing 900 μl of isopropanol. The tubes were mixed by inversion and incubated at -20°C overnight. Following centrifugation at 13,000×g for 5 min, the supernatant was discarded and the DNA pellet washed with 600 μl of 70% ethanol. Centrifugation was repeated a second time and the residual ethanol was removed. The DNA pellet was then allowed to dry briefly before dissolving it in 100 μl DNase/RNase free water (Sigma-Aldrich, Munich, Germany) at 4°C overnight. Concentration and purity of the DNA samples were assessed as described above.

4.2.4 ITS1 nested PCR

In order to confirm the presence of *N. caninum* DNA in the laboratory-maintained isolates and clinical samples as well as to exclude contamination of the negative control (DNA isolated from uninfected VERO cells), the *N. caninum* ITS1 nested PCR was carried as previously described (Bartley *et al.*, 2013b, Buxton *et al.*, 1998). PCRs were run in triplicate and the PCR products were electrophoresed on a 1.8% (w/v)

agarose/TAE gel (Appendix 1.1.3) incorporating Gel Red™ (Biotum, Hayward, CA, USA) and visualised under UV light. A 1kb DNA ladder (Promega, Madison, WI, USA) was used to identify the band (~ 297 bp) indicative of *N. caninum* ITS1 amplification.

4.2.5 Microsatellite markers

Twelve microsatellite markers were selected amongst a broader panel of candidate *loci* that showed length polymorphisms in previous studies (Katzer and Bartley, unpublished).

The identification of these *loci* was carried out by screening a large number of *N. caninum* expressed sequence tags (ESTs) for repetitive sequences, using the Tandem Repeats Finder software (Benson 1999). ESTs derived from tachyzoite cDNA libraries of the Nc-Liverpool isolate and were screened before the complete assembly and annotation of the parasite genome was made publicly available (Katzer, unpublished). In the present study, chromosomal localisation, repeat motif sequence and length of each microsatellite *locus* studied were cross-checked using the *N. caninum* Liverpool genome data that are currently available. Nomenclature of the microsatellite markers used and their chromosome location are shown Table 4.2. Furthermore, all the microsatellites used in the present study were compared with those previously described in other studies (Al-Qassab *et al.*, 2009, Basso *et al.*, 2009, Regidor-Cerrillo *et al.*, 2006) showing that they have not been described to date.

Table 4.2 – Attributes of the *N. caninum* microsatellite *loci* used in this study (source *Neospora caninum* Liverpool genome <https://www.ncbi.nlm.nih.gov/genbank/> and <http://www.toxodb.org>). Chr.: chromosome.

Marker name	Chr. No.	Repeat	Sequence ID (position within chromosome)
MRI_002	VIIa	(CTAT) ₁₁	FR823388.1 2541020-2541400
MRI_007	XI	(TACA) ₁₄ -TACG-(TACA) ₁₀ -(AT) ₇	FR823392.1 2826333-2826623 (NCLIV_056440)
MRI_014	VIIa	(TCTA) ₁₁ -(TA) ₁₀	FR823388.1 2267513-2267897
MRI_016	VIII	(AT) ₉ -(TA) ₂ -(AT) ₉	FR823390.1 6000078-6000405
MRI_027	Ia	(ATAC) _{14M}	FR823380.1 335498-335865
MRI_030	IV	(TAC(A/G)) ₄₀	FR823384.1 1827518-1827790
MRI_036	VIII	(AT) ₁₄	FR823390.1 214889-215138
MRI_037	VI	(TATG) ₁₁	FR823387.1 1319153-1319525
MRI_040	II	(AT) ₁₆	FR823382.1 1200461-1200685
MRI_041	IX	(A(T/C)) ₉₄	FR823385.1 5208444-5208693
MRI_042	Ib	(TAGA) ₁₅ -(TA) ₁₁	FR823381.1 1663036-1663277
MRI_045	Ib	(AT) ₉ -(TAGA) ₁₅ -(AT) ₉	FR823381.1 119905-120195

Based on the DNA sequences flanking the repeat region of each marker, internal and external primer pairs were designed to achieve nested PCR amplification (Table 4.3). Primers were designed using the Primer3 software (version 4.0.0) (Untergasser *et al.*, 2007) ensuring that the amplicon did not exceed 600 bp which is generally considered the upper limit for CE reproducibility in most capillary electrophoresis platforms (Nadon *et al.*, 2013). The 5' end of each internal forward primer was labelled with 6-carboxyfluorescein (6-FAM). Both 6-FAM-labelled and unlabelled PCR primers were synthesised by Eurofins MWG Operon (Eurofins Genomics, Ebersberg, Germany).

4.2.6 Nested PCR of repetitive DNA targets

The reaction master mix, used during both the first and second rounds of nested PCR (nPCR), consisted of 2.5 µl 10× NH₄-based Reaction Buffer, 0.75 µl 50mM MgCl₂, 0.25 µl 10mM dNTP Mix and 2.5 units of BioTaq DNA Polymerase (all Bioline, London, UK), 0.2 µM of forward and reverse primers and DNase/RNase free water to

a final volume of 23 μ l per reaction. Approximately 0.2 μ g/reaction (2 μ l of the 0.1 μ g/ μ l stock) of template or control DNA or 2 μ l of DNase/RNase free water (blank control) were added to the master mix.

A positive *N. caninum* (NC-1 strain) control was added. Negative controls included DNA isolated from uninfected VERO cells and *T. gondii* (RH strain) tachyzoites.

PCRs were performed in a G-Storm thermocycler (G-Storm, Somerton, UK). Initial denaturation (95°C for 5 min) was followed by 40 cycles of denaturation (95°C for 1 min), annealing (from 55 to 59°C, depending on the set of primers, for 1 min) and elongation (72°C for 1 min) with a final extension at 72°C for 5 min. Optimum annealing temperatures were determined by gradient PCR reactions for each set of primers (Table 4.3).

The nPCR products from the primary reaction were diluted 1:100 with DNase/RNase free water and used as a template (2 μ l/reaction) for the secondary round.

PCR products were separated by electrophoresis on 1.8% (w/v) agarose/TAE gel (Appendix I) incorporating Gel Red™ (Biotum, Hayward, CA, USA), using a 1kb DNA ladder (Promega, Madison, WI, USA) as molecular marker. Gels were visualised under UV light and the intensity of each band was visually assessed and scored as weak, intermediate or strong. Based on band intensity scoring, the nPCR products were diluted 1:50 (weak), 1:100 (intermediate) or 1:200 (strong) in deionised DNase/RNase free water (Sigma-Aldrich, Munich, Germany) before submission for fragment size analysis. PCR products producing very faint bands were submitted undiluted.

Table 4.3 – External and internal nPCR primers and annealing temperatures used to amplify the *N. caninum* microsatellite loci.

Marker	External PCR primers (5'-3')		Internal PCR primers (5'-3')		T _a (°C)	Amplicon size (bp)
	Forward	Reverse	Forward	Reverse		
MRI_002	AACGAACGCGTCAGAAGAAC	GCAAGGAAAGCTCGAGACAC	*ATCCTACCCCGATCTCGACT	CACGCAGCGTCTGTGTAGAT	58	381
MRI_007	GGCGTCAATTCCAGAAAAAT	CAGCCTCGCTTTCACCTACT	*AAACACGCTTCTTCACACCA	TCAATGGCATAGATAGCTACGTT	58	291
MRI_014	CCAGACGCCTCTGTATGTGA	GACCCAAAACCGAACAGAAA	*ACATCCACACTCGTGCGTTA	GACACAGCTTTGCCATCTTG	58	385
MRI_016	TAGTACCTTGTGCCGCAAT	TTGCTCTTCTCCCTGCT	*GTCTCTGTTGACCCCTCAGC	CACCTCGTCACTCCCATGTA	57	328
MRI_027	GGCCCGTCTCTAAATGAGT	TTAAGGCCGACCCTTACTG	*TGGCGTCTACACTGTGATCC	CGTCTGCACGTATGTCCGTA	57	369
MRI_030	TGCACACATGCATCTCGTAT	AAGGCTGACGTTAGGGAACA	*ATATTCGTGCATCCATTTCG	TCCGGGCAGATTACTGTTTT	55	273
MRI_036	TGTATGCGCTTATCTGCACCT	TGCAACGACAACACTACACG	*CTGTGCGACGCCTACACATA	AAATACCCACACGCAGCATC	56	250
MRI_037	CAATCGACATGTAGCCGTTT	GAGCGACCAATCTCGTCTTC	*TCGTGTCTCCCCATTCTAA	AAAACGAGAGAAAAGCCGTC	56	373
MRI_040	GAAAGCCACCTGAGGATCT	TCGACACATACGCCATCTA	*ACTATTCGGGGGACGGTAAG	CCGACGCTTGCTACCTATTC	57	225
MRI_041	AGCCACGCTTTTACCTACC	CCAAAGTAACACGCTTCCAA	*GCACCTCAAACACACACAA	CACCTCGCATCACGTTTATA	58	250
MRI_042	ACACGGAAGAGAGCCTAGCA	TTCGTTACGGACAAGACAC	*GCATTCACCTTTGTCCGTGT	AACAGGAATGCCTCCAAGTG	57	242
MRI_045	CCAGTGCATTCACAGAGGTG	GAAAGACAACGAGGGACGAA	*GGCACCTGCTTACTCACGTC	AATGCTCGTCTTTCGCTCTC	57	291

* Internal primers labelled with FAM at the 5' end. T_a: annealing temperature

4.2.7 Automated allele sizing and fragment analysis

The size of the 6-FAM-labelled PCR products was determined by capillary electrophoresis (CE) using a 48-capillary ABI3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) with the Genescan™ ROX500™ Size Standard (Applied Biosystems) at the University of Dundee (DNA Sequences and Services, Medical Science Institute, the University of Dundee). The electrophoretograms were then analysed using both the STRand (<https://www.vgl.ucdavis.edu/informatics/strand>) and the Peak Scanner (Applied Biosystems) software.

Allele numbers were assigned based on amplicon size as determined by CE and a multilocus fragment typing profile was generated for each DNA sample tested. Unique profiles were given a multilocus genotype (MLG) number.

4.2.8 Sequencing of microsatellite markers

Representative alleles in each *locus* were sequenced to ensure that the size variation observed between different alleles was due to differences in repeat unit copy numbers and that the regions flanking each repeat *locus* were conserved. In addition, sequencing enabled the characterisation of each allele based on the number of repeat units. Assessment of the microsatellite markers by sequencing was carried out as outlined by Nadon and colleagues (2013).

PCR products were purified using a Wizard® SV gel and PCR Clean-up system (Promega, Madison, WI, USA) as detailed by the manufacturer. DNA sequencing, using both forward and reverse primers, was performed by Eurofins Genomics (Ebersberg, Germany). For each allele sequenced, a consensus sequence was obtained using the Lasergene Seqman Pro software (version 12) (DNASTAR, Madison, Wisconsin, USA). Consensus sequences of the different alleles of each microsatellite marker were then aligned for detecting length polymorphisms in the repeat regions. In case of failure or poor sequence quality, sequencing was repeated following cloning of the target amplicons.

4.2.8.1 Cloning

The purified PCR product was cloned using the pGEM®-T Easy Vector System (Promega, Madison, WI, USA). Cloning and *E. coli* JM109 transformation were carried out as described in Chapter 2 (2.2.4.1).

The plasmid DNA obtained from transformed bacteria was sequenced using the standard sequencing primers SP6-promoter and T7-promoter (Eurofins Genomics, Ebersberg, Germany). Consensus sequences were obtained as described above.

4.2.9 Specificity

The ability of each set of internal primers to exclusively amplify *N. caninum* DNA was initially assessed using primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to verify that primers did not align to other sequences of related species deposited in GenBank.

Specificity was then experimentally evaluated by testing DNA samples from the related apicomplexan *H. heydornii* (isolate Giessen-1999), *T. gondii* (RH strain), *S. cruzi*, and *B. besnoiti* (Evora strain) in each *locus*-specific nPCR. The DNA samples originated from *H. heydornii* oocysts, *S. cruzi* bradyzoites isolated from bovine tissue cysts and *T. gondii* and *B. besnoiti* tachyzoites cultured *in vitro*.

DNA samples extracted from *H. heydornii* and *B. besnoiti* were kindly provided by Dr Gereon Schares (Friedrich Loeffler Institute, Greifswald, Germany).

4.2.10 Data analysis

4.2.10.1 Typeability of microsatellite markers

Typeability, defined as the proportion of samples that were successfully assigned an allele number or a multilocus microsatellite genotype, was calculated for each microsatellite *locus* as the number of samples in which it was possible to assign an allele number divided by the total number of samples tested (Hotchkiss *et al.*, 2015). Typeability of the multilocus genotyping method was also calculated.

4.2.10.2 Discriminatory power (Simpson's Index of Diversity)

The discriminatory power of a typing method is its ability to distinguish between unrelated samples. In this study, the Simpson's Index of Diversity (SID), calculated using V-DICE (Variable Number Tandem Repeat and Confidence Extractor; <http://www.hpa-bioinformatics.org.uk/cgi-bin/>), was used to evaluate both the discriminatory ability of each individual microsatellite marker and the overall discriminatory power of the multilocus genotyping method. Based on the number of alleles and their relative frequencies, the SID provides an estimate of the probability that two epidemiologically-unrelated samples, randomly selected from the study population, will be attributed to different typing groups (Hunter and Gaston 1988). The SID ranges from 0 to 1, where 1 indicates that all samples analysed have different genotypes and 0 indicates that all samples have identical genotypes.

4.2.10.3 Genetic and genotypic diversity

Indexes of genetic diversity were calculated to describe the reference population of laboratory-maintained *N. caninum* isolates and the study population of DNA samples from bovine abortion cases collected from a specific geographic region of Scotland. Only the samples that were allocated an allele at all 12 *loci* and therefore a MLG were included in the analysis.

Allelic richness and frequency were calculated using the FSTAT software (version 2.9.3.2) (<http://www2.unil.ch/popgen/softwares/fstat.htm>) (Goudet 1995).

The laboratory-maintained isolates with their worldwide origins were considered as the reference population and compared to the *N. caninum* population studied through the analysis of bovine abortion samples collected from a defined area of Scotland. Due to unequal sample size, allelic richness was corrected by standardisation to the smallest sample size in each dataset, corresponding to 8 individual samples.

Nei's unbiased genetic diversity (H_{Nei}) (Nei 1978) was measured to assess polymorphisms in each microsatellite *locus* using FSTAT.

Genotypic diversity (G) was calculated as the number of unique MLGs observed (g) divided by the total number of individual DNA samples genotyped (n): $G=g/n$ (Regidor-Cerrillo *et al.*, 2013). The genotypic diversity was determined for the entire

sample of DNA samples, the reference population (laboratory-maintained isolates) and the study population (clinical samples from bovine abortions).

4.2.10.4 Clustering analysis

The multilocus microsatellite profiles obtained using the 12 markers were used to build a neighbour-joining dendrogram using ClustalW2 Phylogeny (https://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The dendrogram was then visualised using the software FigTree v. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). Only isolates that were amplified at all 12 *loci* were included in the analysis.

4.3 Results

4.3.1 Characterisation of microsatellite markers and allele assignment

BLAST searches on ToxoDB (<http://www.toxodb.org>) revealed that the 12 microsatellite *loci* used in this study were located on 9 (Ia, Ib, II, IV, VI, VIIa, VIII, IX and XI) of the 14 chromosomes of the *N. caninum* genome. The microsatellite marker pairs MRI_002 and MRI_014, MRI_016 and M_036 and MRI_042 and MRI_045 were located within the same chromosome: VIIa, VIII and Ib respectively (Table 4.2). All microsatellite *loci* were in non-coding regions. MRI_007 was included within an intron sequence of the gene NCLIV_056440 while the other markers were situated in intergenic regions.

Twelve nPCRs were developed using the internal and external primer pairs shown in (Table 4.3). Development and optimisation of the nPCRs and preliminary characterisation of the microsatellite markers were carried out using DNA samples from the 14 laboratory-maintained isolates. Amplification of the 12 microsatellite markers was successfully achieved in these samples. Besides showing the presence of a single band for each marker in each sample analysed, agarose gel electrophoresis of the nPCR products revealed noticeable length polymorphisms amongst amplicons within the same target *locus*. This provided a preliminary indication of the presence of different alleles characterised by different fragment lengths (Figure 4.1).

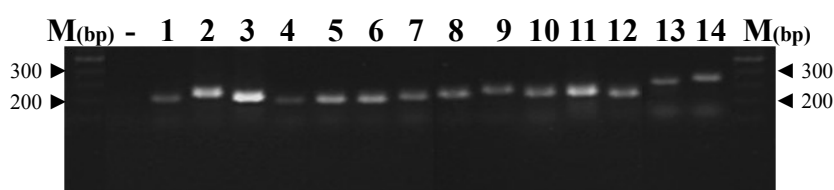


Figure 4.1 – Agarose gel showing fragment size polymorphisms of the MRI_030 microsatellite marker amongst *N. caninum* laboratory-maintained isolates. -: negative control (DNA isolated from uninfected Vero cells); 1: BPA-1, 2: Nc-Freiburg02, 3: Nc-Freiburgh03, 4: Nc-GER1, 5: Nc-Porto; 6: Nc-Spain2; 7: Nc-Drachten; 8: JPA-2, 9: JPA-4, 10: Nc-SweB1, 11: NcBeef; 12: Poland, 13: Nc-LivB1; 14: NC-1. M(bp): molecular marker (base pairs).

Automated CE analysis of FAM-labelled nPCR products resulted in electropherograms that consisted of a single peak of fluorescence. For each *locus*, alleles were assigned based on this main peak corresponding to the size of the amplicon.

Additional peaks differing from the primary peak by 2-4 bp were sporadically observed, these showed a considerably lower fluorescence and were attributed to strand slippage of the *Taq* polymerase on the microsatellite sequence (stutter peaks). In previous studies, secondary peaks with a height $\geq 30\%$ of the primary peak were considered indicative of the presence of multiple genotypes within the same sample (Hotchkiss *et al.*, 2015). Based on this arbitrary threshold, no evidence of mixed *N. caninum* genotypes was found in individual DNA samples.

Direct sequence analysis was carried out on at least one representative of each allele identified.

Allele size measured by automated fragment analysis was compared with the size obtained by direct sequence analysis for each allele identified. Discrepancies between fragment and sequence analysis were observed; however, these were consistent across the entire size range at each *locus*. Repeated nPCR and fragment analysis of representative alleles at each *locus* confirmed the consistency and repeatability of the sizing method (not shown).

Sequencing of the nPCR products confirmed that the size variations observed at each *locus* derived from differences in the copy number of repeated units. In addition, the nature of the repeated motifs and the conservation of the regions flanking the repeat sequence were also verified. The variable repeats observed were either dinucleotide or tetranucleotide. Dinucleotide repeats were predominantly (AT)_n (Table 4.4).

Linear regression analysis showed that, within the panel of microsatellite markers examined in this study, the weak correlation ($R^2 = 0.281$) between the length of the repeat and the number of alleles identified in each *locus* was not statistically significant ($p = 0.075$) (linear regression analysis was performed in Microsoft Excel, version 15.28).

The microsatellite markers MRI_002, MRI_027, MRI_030, MRI_036, MRI_037, MRI_040 and MRI_041 were characterised by polymorphism of individual repeat units whereas length variations in more than one tandemly repeated sequence were

found within the microsatellite markers MRI_007, MRI_014, MRI_016, MRI_042 and MRI_045. MRI_007 and MRI_045 had 3 variable dinucleotide or tetranucleotide repeat units within their sequences. MRI_014 and MRI_042 were both characterised by one tetranucleotide and one dinucleotide variable repeat: (TCTA)_n and (TA)_n, (TAGA)_n and (TA)_n respectively (Table 4.4).

In MRI_016, 2 variable (AT)_n repeats were separated by a single cytosine. In addition, a single nucleotide polymorphism (SNP) was detected in allele 7.

In MRI_030, the variable repeat unit (TACA)_n was intercalated with TACG motifs. Similarly, substitutions of the thiamine (T) with a cytosine (C) in the (AT)_n repeats of MRI_041 were observed.

After preliminary validation on laboratory-maintained *N. caninum* isolates the microsatellite analysis was applied to DNA samples extracted from clinical samples. Amplification of all microsatellite markers was not achieved in all clinical samples (see Typeability 4.3.2).

Nomenclature, repeated motif and size determined by direct sequencing and CE for each allele identified in this study are summarised in Table 4.4.

Table 4.4 – Allele number allocations and sizes measured by sequence analysis and automated fragment sizing (CE) for each of the 12 microsatellite markers (table continued overleaf).

Locus	Repeat	Allele No.	Amplicon size sequencing (bp)	Amplicon size CE (range)
MRI_002	(CTAT) ₁₁	1	381	382-383
	(CTAT) ₁₂	2	385	386-387
	(CTAT) ₁₃	3	389	390-391
	(CTAT) ₁₄	4	393	394-395
	(CTAT) ₁₅	5	397	398-399
	(CTAT) ₁₆	6	401	402-403
	(CTAT) ₁₇	7	405	406-407
	(CTAT) ₁₈	8	409	410-411
	(CTAT) ₁₉	9	413	414-415
MRI_007	(TACA) ₁₄ -TACG-(TACA) ₁₀ -(AT) ₇	1	283	283.5-284.5
	(TACA) ₁₅ -TACG-(TACA) ₁₀ -(AT) ₇	2	287	287.5-288.5
	(TACA) ₁₄ -TACG-(TACA) ₁₂ -(AT) ₇	3	291	291.5-292.5
	(TACA) ₁₅ -TACG-(TACA) ₁₂ -(AT) ₇	4	295	295.5-296.5
	(TACA) ₁₆ -TACG-(TACA) ₁₂ -(AT) ₆	5	297	297.5-298.5
	(TACA) ₁₈ -TACG-(TACA) ₁₄ -(AT) ₆	6	313	313.5-314.5
	(TACA) ₁₉ -TACG-(TACA) ₁₄ -(AT) ₇	7	319	319.5-320.5
	(TACA) ₂₁ -TACG-(TACA) ₁₅ -(AT) ₆	8	329	329.5-330.5
MRI_014	(TCTA) ₆ -(TA) ₇	1	363	364-365
	(TCTA) ₆ -(TA) ₉	2	367	368-369
	(TCTA) _x -(TA) _y	3	NS	370-371
	(TCTA) _x -(TA) _y	4	NS	374-375
	(TCTA) ₉ -(TA) ₁₀	5	377	378-379
	(TCTA) ₁₁ -(TA) ₉	6	383	384-385
	(TCTA) ₁₁ -(TA) ₁₀	7	385	386-387
	(TCTA) ₁₂ -(TA) ₁₃	8	395	390-391
	(TCTA) _x -(TA) _y	9	NS	332-333
MRI_016	(AT) ₈ -C-(AT) ₁₀	1	326	326.5-327.5
	(AT) ₁₁ -C-(AT) ₉	2	330	330.5-331.5
	(AT) ₁₂ -C-(AT) ₉	3	332	332.5-333.5
	(AT) ₁₂ -C-(AT) ₁₀	4	334	334.5-335.5
	(AT) ₁₂ -C-(AT) ₁₁	5	336	336.5-337.5
	(AT) ₁₅ -C-(AT) ₁₀	6	340	340.5-341.5
	(AT) ₁₆ -C-(AT) ₁₁ ^{SNP}	7	342	342.5-343.5
MRI_027	(ATAC) ₆	1	337	336.5-338
	(ATAC) ₇	2	341	341-342.5
	(ATAC) ₈	3	345	345-346.5
	(ATAC) ₉	4	349	348.5-349.5
	(ATAC) ₁₄	5	365	364.5-366.5
MRI_030	(TAC(A/G)) ₃₀	1	233	232.5-233.5
	(TAC(A/G)) ₃₁	2	237	236.5-237.5
	(TAC(A/G)) ₃₃	3	245	244.5-245.5
	(TAC(A/G)) ₃₄	4	249	248.5-249.5
	(TAC(A/G)) ₃₅	5	253	252.5-253.5
	(TAC(A/G)) ₃₆	6	257	256.5-257.5
	(TAC(A/G)) ₃₈	7	265	264.5-265.5
	(TAC(A/G)) ₃₉	8	269	268.5-269.5
	(TAC(A/G)) ₄₁	9	277	276.5-277.5
MRI_036	(AT) ₁₀	1	242	242.5-243.5
	(AT) ₁₃	2	248	248.5-249.5
	(AT) ₁₄	3	250	250.5-251.5
	(AT) ₁₅	4	252	252.5-253.5
	(AT) ₁₇	5	256	256.5-257.5
	(AT) ₁₉	6	260	260.5-261.5
	(AT) ₂₀	7	262	262.5-263.5

Locus	Repeat	Allele No.	Amplicon size sequencing (bp)	Amplicon size CE (range)
MRI_036	(AT) ₂₂	8	266	266.5-267.5
	(AT) ₃₂	9	286	286.5-287.5
MRI_037	(TATG) ₁₀	1	369	368-370
	(TATG) ₁₄	2	385	386-387
	(TATG) ₁₅	3	389	390-391
	(TATG) _x	4	NS	395-396
	(TATG) _x	5	NS	400-401
	(TATG) ₁₈	6	403	404-405
	(TATG) _x	7	NS	408-409
	(TATG) _x	8	NS	412-413
MRI_040	(AT) ₁₁	1	217	216.5-217.5
	(AT) ₁₄	2	223	222.5-223.5
	(AT) ₁₅	3	225	224.5-225.5
	(AT) ₁₆	4	227	226.5-227.5
	(AT) ₁₇	5	229	228.5-229.5
	(AT) _x	6	NS	234.5-235.5
	(AT) _x	7	NS	236.5-237.5
	(AT) _x	8	NS	238.5-239.5
	(AT) ₂₃	9	241	240.5-241.5
MRI_041	(A(T/C)) ₈₅	1	232	233-234
	(A(T/C)) ₈₇	2	236	237-238
	(A(T/C)) ₈₈	3	238	239-240
	(A(T/C)) ₈₉	4	240	241-242
	(A(T/C)) ₉₀	5	242	243-244
	(A(T/C)) ₉₁	6	244	245-246
	(A(T/C)) ₉₂	7	246	247-248
	(A(T/C)) ₉₃	8	248	248.5-250
	(A(T/C)) ₉₄	9	250	251-252
	(A(T/C)) ₉₇	10	256	257-258
	(A(T/C)) ₉₈	11	258	258.5-260
MRI_042	(TAGA) ₁₂ -(TA) ₉	1	226	227-228
	(TAGA) _x -(TA) _y	2	NS	233-234
	(TAGA) _x -(TA) _y	3	NS	237-238
	(TAGA) ₁₅ -(TA) ₉	4	238	239-240
	(TAGA) ₁₅ -(TA) ₁₀	5	240	241-242
	(TAGA) ₁₅ -(TA) ₁₁	6	242	243-244
	(TAGA) _x -(TA) _y	7	NS	245-246
	(TAGA) _x -(TA) _y	8	NS	247-248
	(TAGA) ₁₇ -(TA) ₁₁	9	250	251-252
	(TAGA) ₁₈ -(TA) ₁₂	10	256	257-258
MRI_045	(AT) ₈ -(TAGA) ₇ -(AT) ₉	1	257	258-259
	(AT) _x -(TAGA) _y -(AT) _z	2	NS	260-261
	(AT) _x -(TAGA) _y -(AT) _z	3	NS	262-263
	(AT) ₉ -(TAGA) ₈ -(AT) ₉	4	263	264-265
	(AT) _x -(TAGA) _y -(AT) _z	5	NS	266-267
	(AT) _x -(TAGA) _y -(AT) _z	6	NS	268-269
	(AT) ₉ -(TAGA) ₉ -(AT) ₁₀	7	269	270-271
	(AT) _x -(TAGA) _y -(AT) _z	8	NS	274-275
	(AT) _x -(TAGA) _y -(AT) _z	9	NS	276-277
	(AT) ₉ -(TAGA) ₁₅ -(AT) ₉	10	291	287-292

NS: sequence not analysed due to poor sequencing quality.
 SNP: single nucleotide polymorphism.

4.3.2 Typeability

All 14 laboratory-maintained *N. caninum* isolates were assigned an allele at all 12 microsatellite *loci*. Fourteen unique multilocus profiles were obtained and allocated a MLG number (Table 4.5). Therefore, in these samples, the typeability of each individual marker as well as of the multilocus microsatellite typing tool was 1.0 (14/14, 95%CI: 0.77-1.00).

Within the clinical samples from bovine abortion cases, only 8 out of 28 *N. caninum* ITS1 nPCR positive samples were conclusively assigned a MLG (Table 4.6).

The remaining 20 samples had at least one marker which could not be typed due to non-amplification during nested PCR or, less frequently, failure of automated fragment sizing that resulted in inconclusive electropherograms (Table 4.6).

The typeability of the genotyping method was 0.28 (8/28, 95%CI: 0.15-0.47). At the marker level, the typeability ranged from 0.96 (95%CI: 0.82-0.99) (MRI_002, MRI_007, MRI_016 and MRI_027) to 0.43 (12/28, 95%CI: 0.26-0.60) (MRI_045) (Table 4.6).

The typeability values of each microsatellite marker and the multilocus microsatellite typing tool when applied to laboratory-maintained isolates and clinical samples are summarised in Table 4.7.

4.3.3 Specificity

Based on the primers-BLAST results, none of the primers used in nested PCR showed significant alignment to non-*N. caninum* apicomplexan species.

Experimentally, the absence of amplification products from *H. heydornii*, *T. gondii*, *S. cruzi*, and *B. besnoiti* DNA samples indicated that the nPCRs developed for the amplification of the 12 *N. caninum* microsatellite markers were specie-specific (Figure 4.2).



Figure 4.2 – Agarose gel showing absence of amplification of the MRI_030 microsatellite marker from DNA samples of *H. heydornii* (2a-2b), *T. gondii* (3a-3b), *S. cruzi* (4a-4b) and *B. besnoiti* (5a-5b); 1a-1b: *N. caninum* BPA-1 positive control, 6a-6b: *N. caninum* Nc-Freiburg03 positive control, -: negative control (DNA isolated from uninfected Vero cells). M(bp): molecular marker (base pairs).

Table 4.5 Multilocus microsatellite genotyping of the *N. caninum* laboratory-maintained isolates ($n=14$).

Isolate	MRI_002	MRI_007	MRI_014	MRI_016	MRI_027	MRI_030	MRI_036	MRI_037	MRI_040	MRI_041	MRI_042	MRI_045	MLG
NC-1	1	2	6	2	5	9	3	1	4	9	5	10	1
BPA1	4	8	6	1	2	5	5	2	7	10	6	3	2
JAP-2	4	8	6	1	2	5	7	2	7	11	9	3	3
JAP-4	4	8	6	1	2	8	5	2	7	10	5	3	4
Nc-Beef	9	3	4	6	3	6	1	5	1	8	7	4	5
Nc-Drachten	2	7	7	6	1	2	6	9	7	2	8	1	6
Nc-Freiburg02	6	1	7	5	2	7	4	3	1	3	2	4	7
Nc-Freiburg03	9	4	3	5	2	5	4	4	7	1	9	6	8
Nc-GER 1	3	5	3	4	4	3	4	7	5	5	3	8	9
Nc-LivB1	7	1	8	3	3	9	2	7	2	8	1	7	10
Nc-Poland	2	6	9	6	1	2	7	9	9	11	10	1	11
Nc-Porto	1	7	9	6	1	1	8	9	6	2	9	1	12
Nc-Spain2	2	6	9	6	1	2	7	9	7	2	6	2	13
Nc-SweB1	2	6	4	7	4	6	2	4	6	4	4	1	14

MLG: Multilocus genotype.

Table 4.6 *N. caninum* microsatellite alleles found in clinical samples from the Dumfries and Galloway region of Scotland and multilocus genotypes (MLGs).

Herd no.	Foetus ID no.	Tissue	Date	ITS1 nPCR	MRI_002	MRI_007	MRI_014	MRI_016	MRI_027	MRI_030	MRI_036	MRI_037	MRI_040	MRI_041	MRI_042	MRI_045	MLG
1	C421441	brain	Mar-08	+++	5	4	2	5	1	5	9	6	7	8	7	NA	
2	C425656	brain	Sep-09	+++	7	3	1	5	1	NA	NA	NA	3	6	4	6	
	C425673	brain	Sep-09	++-	7	4	4	5	1	NA	9	NA	3	7	4	6	
3	C421179	brain	Apr-08	+++	4	4	5	5	2	3	9	6	8	9	7	5	15
4	C426552	brain	Mar-10	+++	6	5	NCE	NA	1	4	9	6	7	8	5	NA	
5	C426622	brain	Mar-10	+++	5	3	1	5	1	3	9	6	7	4	5	6	16
	C426628-1*	brain	Mar-10	+++	5	3	1	5	1	3	9	6	7	4	5	6	16
	C426628-2*	brain	Mar-10	++-	5	3	1	5	1	3	9	6	7	4	5	6	16
	C426655	brain	Mar-10	+++	5	3	NA	5	1	3	9	NA	NA	4	5	NA	
	C426704	brain	Mar-10	+--	5	3	1	5	1	NA	9	6	NA	4	NA	NA	
	C428452-1*	heart	Jun-11	+++	5	5	2	5	1	5	9	2	7	4	5	6	17
	C428452-2*	placenta	Jun-11	+++	5	5	2	5	1	NA	NCE	2	NA	4	5	NA	
	C428848	heart	Jun-11	+++	5	3	2	5	1	3	9	NA	NA	NA	NA	NCE	
	C431174-1*	brain	Jun-11	+++	5	3	2	5	1	3	NA	NA	7	4	5	NCE	
6	C431174-2*	brain	Jun-11	+++	5	3	2	5	1	3	NA	NA	NA	NA	NA	NA	
	C431181	brain	Jun-11	+++	5	3	2	5	1	3	9	9	7	NA	NA	NA	
	C422245	brain	Aug-08	+++	6	8	1	5	4	4	9	8	5	7	5	9	18
7	C421369	brain	Apr-08	+++	7	4	1	4	1	3	9	6	7	8	3	6	19
8	C423210	heart	Dec-08	+++	8	3	1	5	3	NA	9	9	6	7	8	NA	
	C423154	brain	Dec-08	++-	5	4	1	4	1	3	9	9	6	7	8	NA	
9	C422401-1*	brain	Aug-08	++-	7	3	3	5	1	3	9	NA	7	6	7	NA	
	C422401-2*	heart	Aug-08	+--	NA	NA	3	5	1	3	9	NA	NA	6	7	NA	
10	C425723	brain	Sep-09	+++	6	3	1	5	1	3	9	7	NA	8	8	6	
	C423800	brain	Mar-09	++-	6	3	NA	5	NA	NA	9	7	NA	8	NA	NA	
11	C421682	brain	Jun-08	+++	7	5	1	5	1	4	9	9	9	7	7	7	20
	C423152	brain	Dec-08	+++	7	5	1	5	1	3	NA	NA	9	NA	NA	NA	
12	C426057	brain	Dec-08	+++	7	4	1	5	1	4	9	NA	NA	8	7	6	
13	C420872	brain	Mar-08	++-	5	3	3	5	1	3	9	9	7	NA	NA	NCE	

* Foetuses aborted by different dams but submitted the same day. ITS1 nPCR results: +++ 3 positive replicates, ++- 2 positive replicates, +-- 1 positive replicate.

NA: not amplified. NCE: not sized due to poor quality of the CE electrophoretogram. MLG: multilocus genotype.

Table 4.7 – Typeability and discriminatory power of the 12 microsatellite markers analysed. Typeability and Simpson’s Index of Diversity (SID) are reported for allele and multilocus genotype (MLG) assignment with 95% confidence interval (95% CI).

	MRI_002	MRI_007	MRI_014	MRI_016	MRI_027	MRI_030	MRI_036	MRI_037	MRI_040	MRI_041	MRI_042	MRI_045	MLG	
Laboratory-maintained isolates	Typeability (95%CI) (n=14)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.00 (0.77-1.00)	1.0 (0.77-1.00)	1.00 (0.77-1.00)	
	SID (95%CI) (n=14)	0.82 (0.73-0.90)	0.85 (0.78-0.91)	0.81 (0.73-0.88)	0.79 (0.66-0.91)	0.75 (0.64-0.85)	0.85 (0.78-0.91)	0.85 (0.78-0.91)	0.82 (0.73-0.90)	0.75 (0.59-0.92)	0.87 (0.81-0.92)	0.87 (0.79-0.94)	0.83 (0.73-0.92)	1.00 (0.96-1.00)
Clinical samples	Typeability (95%CI) (n=28)	0.96 (0.82-0.99)	0.96 (0.82-0.99)	0.89 (0.73-0.96)	0.96 (0.82-0.99)	0.96 (0.82-0.99)	0.79 (0.59-0.91)	0.82 (0.63-0.94)	0.64 (0.44-0.81)	0.75 (0.55-0.89)	0.82 (0.63-0.94)	0.75 (0.55-0.89)	0.43 (0.26-0.60)	0.28 (0.15-0.47)
	SID (95%CI) (n=28)	0.64 (0.51-0.77) (n=27)	0.61 (0.47-0.74) (n=27)	0.63 (0.50-0.77) (n=25)	0.13 (0.00-0.30) (n=27)	0.21 (0.01-0.41) (n=27)	0.43 (0.21-0.64) (n=22)	0.00 (0.00-0.02) (n=23)	0.70 (0.56-0.83) (n=18)	0.63 (0.41-0.84) (n=19)	0.74 (0.67-0.81) (n=23)	0.70 (0.59-0.81) (n=21)	0.42 (0.09-0.74) (n=12)	0.78 (0.61-0.95) (n=8)
Overall	Typeability (95%CI) (n=42)	0.98 (0.88-0.99)	0.98 (0.88-0.99)	0.93 (0.81-0.97)	0.98 (0.88-0.99)	0.98 (0.88-0.99)	0.86 (0.72-0.93)	0.88 (0.75-0.94)	0.76 (0.61-0.86)	0.83 (0.69-0.92)	0.88 (0.75-0.95)	0.83 (0.69-0.92)	0.64 (0.49-0.77)	0.52 (0.37-0.66)
	SID (95%CI) (n=42)	0.80 (0.73-0.88) (n=41)	0.77 (0.69-0.86) (n=41)	0.81 (0.74-0.88) (n=39)	0.54 (0.37-0.70) (n=41)	0.50 (0.33-0.67) (n=41)	0.73 (0.60-0.86) (n=36)	0.59 (0.41-0.77) (n=37)	0.81 (0.74-0.88) (n=32)	0.70 (0.55-0.85) (n=33)	0.85 (0.80-0.91) (n=37)	0.84 (0.78-0.90) (n=35)	0.80 (0.68-0.91) (n=27)	0.94 (0.91-0.97) (n=22)

4.3.4 Discriminatory power (SID)

The range of repeat units varied across the 12 microsatellite markers from 5 to 11 allele sizes identified per *locus*. This resulted in a generally high discriminatory power.

Based on the results obtained from the laboratory-maintained *N. caninum* isolates together with the clinical samples, MRI_041 was the most discriminatory marker (SID 0.85; 95%CI: 0.80-0.91) followed by MRI_042 (SID 0.84; 95%CI: 0.78-0.90) and MRI_014 with MRI_037 (both showing a SID of 0.81; 95%CI: 0.74-0.88). In contrast, MRI_027 (SID 0.50; 95%CI: 0.33-0.67), MRI_016 (SID 0.54; 95%CI: 0.37-0.70) and MRI_036 (SID 0.59; 95%CI: 0.41-0.77) were the least polymorphic markers of the panel (Table 4.7).

Nevertheless, all markers showed relatively high discriminatory power (SID \geq 0.75) when the reference population of laboratory-maintained isolates with worldwide origin was considered singularly. However, when the clinical samples were analysed independently, some microsatellite markers such as MRI_036 (SID 0.00; 95%CI: 0.00-0.02), MRI_016 (SID 0.13; 95%CI: 0.00-0.30) and MRI_027 (SID 0.21; 95%CI: 0.01-0.41) showed absent or very limited discriminatory power.

The overall SID of the typing tool based on the 12 markers analysed was 0.94 (95%CI: 0.91-0.97); however, the sample size was limited to the 14 laboratory-maintained isolates and only 8 clinical samples which were assigned a 12-marker MLG. The use of the 6 most informative markers (i.e. those with the highest discriminatory power) characterised by adequate typeability (>0.75) (MRI_002, MRI_007, MRI_014, MRI_037, MRI_041 and MRI_042) would have shown a very similar SID of 0.95 (95%CI: 0.93-0.97). The simpler typing tool based on 6 selected markers showed an increase in typeability: from 0.52 (22/42; 95%CI: 0.38-0.67) to 0.64 (27/42; 95%CI: 0.49-0.77) without compromising the discriminatory power (Table 4.8).

Table 4.8 Simpson’s Index of Diversity (SID) and typeability of the 12-markers genotyping tool and of simpler tool based on 6 selected markers.

Genotyping tool	N* (clinical samples)	MLGs (MLGs in clinical samples)	SID (95%CI)	Typeability (clinical samples) (95%CI)
12 markers	22 (8)	20 (6)	0.94 (0.91-0.97)	22/42 0.52 (8/28 0.28) (0.37-0.66)
6 selected markers¹	27 (13)	24 (10)	0.95 (0.93-0.97)	27/42 0.64 (12/28 0.43) (0.49 – 0.77)

* Number of samples for which a multilocus genotype (MLG) was assigned according to the different typing option.

¹ 6 most discriminatory *loci* characterised by typeability > 0.75: MRI_002, MRI_007, MRI_014, MRI_037, MRI_041, MRI_042.

The selection of this smaller panel of microsatellite markers was carried out based on both SID and typeability. In this study, the low typeability of some markers had a significant impact on the selection of the optimal markers and it is responsible for the exclusion of some discriminatory markers and the inclusion of markers which have lower SID. For example, MRI_045 that showed a relatively high overall SID (0.80; 95%CI: 0.68-0.91) was discarded due to its low overall typeability (0.64; 95%CI: 0.49-0.77). Conversely, MRI_007 that had a slightly lower SID (0.77; 95%CI: 0.69-0.86) was selected as it enabled the typing of most DNA samples (typeability = 0.98; 95%CI: 0.88-0.99) besides having a SID of 0.77 (95%CI: 0.69-0.86) (Table 4.7).

4.3.5 Genetic and genotypic diversity

Frequency and distribution of the predominant alleles varied between the reference population of laboratory-maintained *N. caninum* isolates and the study population of Scottish clinical samples. In the Scottish clinical samples, new alleles of the microsatellite markers MRI_002 (alleles 5 and 8), MRI_014 (alleles 1, 2 and 5), MRI_030 (allele 4), MRI_036 (allele 9), MRI_037 (alleles 6 and 8), MRI_040 (alleles 3 and 8), MRI_041 (alleles 6 and 7) and MRI_045 (alleles 5 and 9), that were not previously observed in the laboratory-maintained isolates, were found (Figure 4.3). Interestingly, at the *locus* MRI_036 all clinical samples in which amplification and fragment sizing were achieved were characterised by the allele 9 which was exclusively detected in this population.

All 12 microsatellite markers were highly polymorphic amongst the laboratory-maintained isolates: the average number of alleles (A) per *locus* was 7.5 ± 1.31 and ranged from 5 (MRI_027) to 10 (MRI_042) ($n=14$). Within the clinical samples, the mean allele number per *locus* was reduced and corresponded to 4.1 ± 1.44 if the complete dataset of the clinical samples ($n=28$) was considered or to 3.25 ± 0.96 if only those samples ($n=8$) that were typed at all *loci* were examined. This resulted in an average allelic richness (Ar) of 6.79 ± 1.04 and 3.8 ± 1.35 (the Ar was calculated based on minimum sample size of 8 individuals) in the reference and study population respectively. Regardless whether the full or partial dataset was considered, the markers MRI_036 and MRI_016 showed absent or limited polymorphism with only 1 and 2 alleles identified respectively within the clinical samples.

The reference population was characterised by extensive genetic diversity showing an average Nei's unbiased genetic diversity value (H_{Nei}) of 0.88 ± 0.04 . Lower yet considerable genetic diversity was observed amongst the clinical samples ($H_{Nei} = 0.55 \pm 0.23$) (Table 4.9).

A , Ar and H_{Nei} per *locus* and population are summarised in Table 4.10.

The same genetic indexes were also calculated based on the multilocus analysis of the 6 microsatellite markers characterised by the highest discriminatory power and typeability > 0.75 . The analysis using this multilocus microsatellite method was carried out considering the 13 samples that were assigned an allele at all 6 markers. Using this typing tool, the observed Ar was 7.82 ± 1.46 and 4.16 ± 0.23 whereas the H_{Nei} was 0.90 ± 0.03 and 0.70 ± 0.10 in the reference and study population respectively (Table 4.9).

Full twelve-*loci* genotypes were obtained in 52.4% (22/42) of the samples analysed (100% or 14/14 of the samples of the reference population and 28.6% or 8/28 of the clinical samples).

All MLGs in the reference population were different ($G=14/14$ or 1.00). Within the clinical samples, the genotypic diversity was 0.62 ($G=6/8$) as 3 samples showed identical MLGs. Interestingly, these three samples originated from the same herd (herd 5) and were collected during a *N. caninum* abortion outbreak in March 2010. Within the complete dataset, 20 of the 22 samples typed at all *loci* showed a unique MLG thus resulting in a high observed genotypic diversity: 0.91.

When the multilocus analysis was carried out considering the 6 selected markers only, the genotypic diversity within the clinical samples increased from 0.62 to 0.77 (G=10/13). Using this method, 4 new MLGs could be discriminated. One additional sample, for which definitive genotyping was not achieved with the 12-markers analysis, had a MLG identical to a previously observed one that was identified in the same herd (herd 5).

Genetic and genotypic diversity indexes assessed with the 12- and 6-markers typing methods for the laboratory-maintained *N. caninum* isolates and the clinical samples are shown in Table 4.9.

In the present study, linkage disequilibrium analysis to investigate the possible non-random association of alleles at different microsatellite *loci* was not performed due to the limited samples size. However, no obvious relationship between alleles at different *loci* was observed.

Table 4.9 – Genetic and genotypic diversity of *N. caninum* in the reference population and the study population when a 12- or 6-*loci* microsatellite analysis is applied.

Typing tool	Population	N* ¹	Number of alleles per locus (A) Mean ± SD	Allelic richness* ² (Ar) Mean ± SD	Genetic diversity (H _{Nei}) Mean ± SD	Genotypic diversity
12 microsatellite markers	Lab isolates (worldwide reference population)	14	7.50 ± 1.31	6.79 ± 1.04	0.88 ± 0.04	1.00 (14/14)
	Clinical samples (Dumfries and Galloway)	8	3.25 ± 0.96	3.25 ± 0.96	0.55 ± 0.23	0.62 (6/8)
6 microsatellite markers¹	Lab isolates (worldwide reference population)	14	7.83 ± 1.47	7.82 ± 1.46	0.90 ± 0.03	1.00 (14/14)
	Clinical samples (Dumfries and Galloway)	13	4.16 ± 0.75	4.16 ± 0.75	0.70 ± 0.10	0.77 (10/13)

¹ MRI_002, MRI_007, MRI_014, MRI_037, MRI_041 and MRI_042.

*¹ Number of samples for which a multilocus genotype (MLG) was assigned using the different typing option.

*² Average allelic richness per population corrected for unequal sample size by standardisation to the smallest sample size in the dataset corresponding to 8 and 13 DNA samples in (6 microsatellite markers analysis).

Table 4.10 Genetic diversity of *N. caninum* per *locus* in the reference population (laboratory-maintained isolates) and the study population (clinical samples from the Dumfries and Galloway area of Scotland). Only samples with a complete 12-markers microsatellite profile were included.

	Population	MRI 002	MRI 007	MRI 014	MRI 016	MRI 027	MRI 030	MRI 036	MRI 037	MRI 040	MRI 041	MRI 042	MRI 045	Mean ± SD
Number of alleles (A)	Laboratory-maintained isolates	7	8	6	7	5	8	8	7	7	9	10	8	7.50 ± 1.31
	Clinical samples	4	4	3	2	3	3	1	4	4	4	3	4	3.25± 0.96
Allelic richness (Ar)*	Laboratory-maintained isolates	6.42	7.23	5.75	6.27	4.77	7.25	7.25	6.42	6.25	8.05	8.72	7.10	6.36 ± 0.97
	Clinical samples	4.00	4.00	3.00	2.00	3.00	3.00	1.00	4.00	4.00	4.00	3.00	4.00	3.25± 0.96
Genetic diversity (H_{Nei})	Laboratory-maintained isolates	0.88	0.91	0.87	0.85	0.80	0.91	0.91	0.88	0.81	0.93	0.94	0.89	0.88 ± 0.04
	Clinical samples	0.75	0.75	0.46	0.25	0.46	0.61	0.00	0.64	0.64	0.75	0.61	0.64	0.55 ± 0.22

H_{Nei}: Nei's unbiased genetic diversity. SD: standard deviation.

* Allelic richness per *locus* and population corrected for unequal sample size by standardisation to the smallest sample in each dataset (8 DNA isolates).

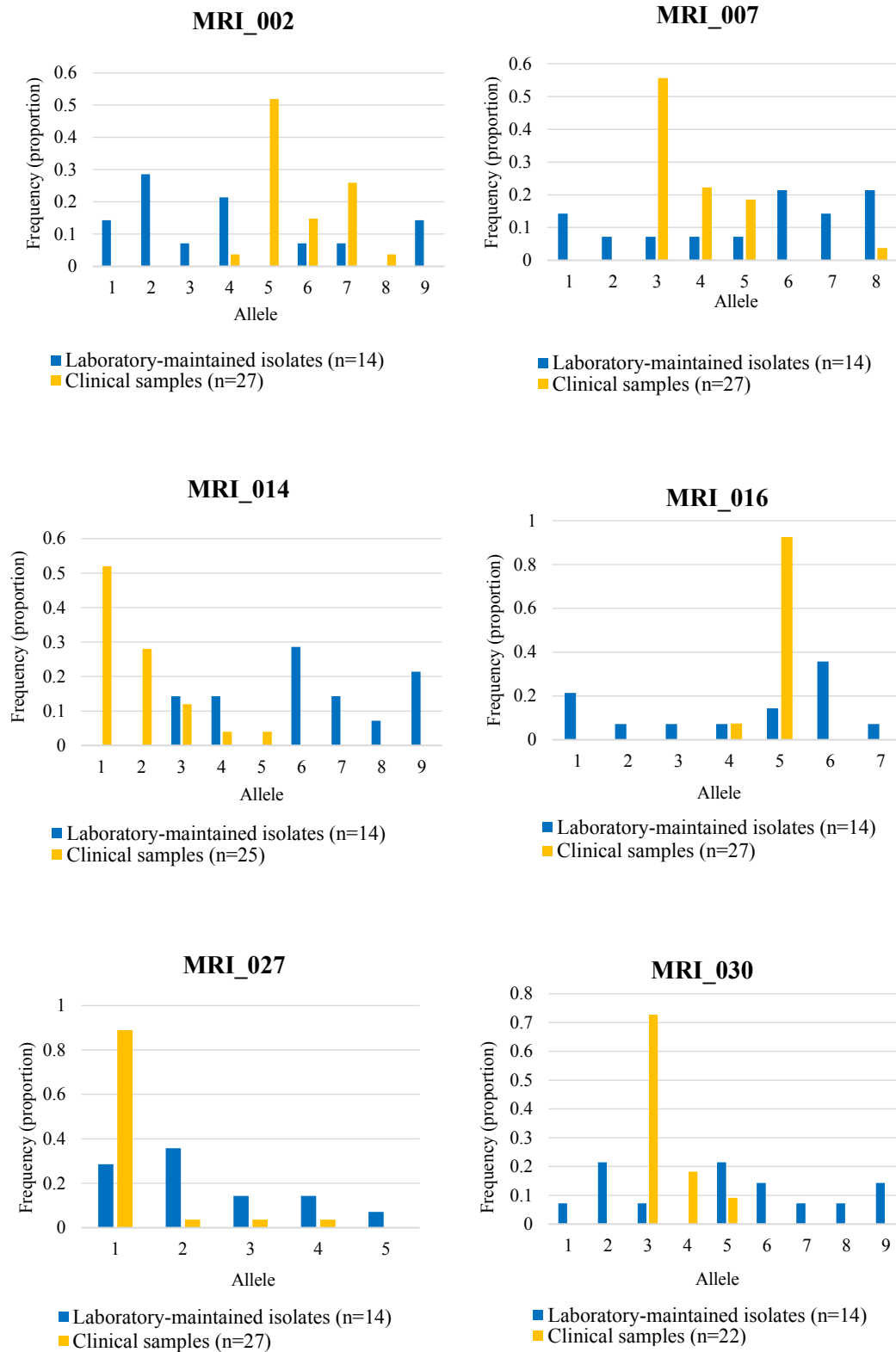
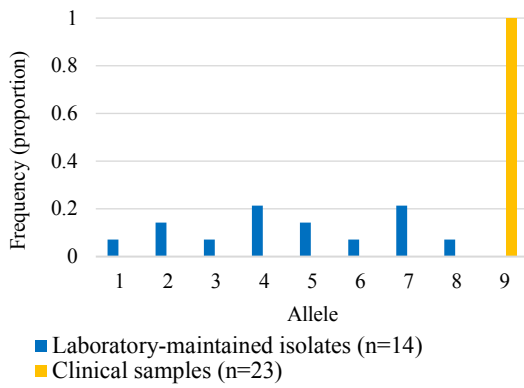
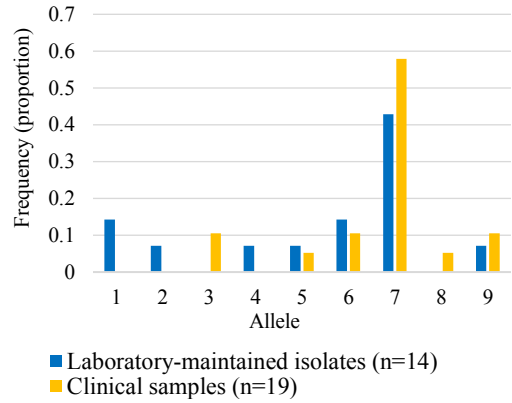


Figure 4.3 – Allele frequency distribution of the 12 microsatellite markers in the laboratory-maintained *N. caninum* isolates with worldwide origin (blue bars) and the clinical samples from bovine abortion cases collected in the Dumfries and Galloway region of Scotland (yellow bars) (figure continues overleaf).

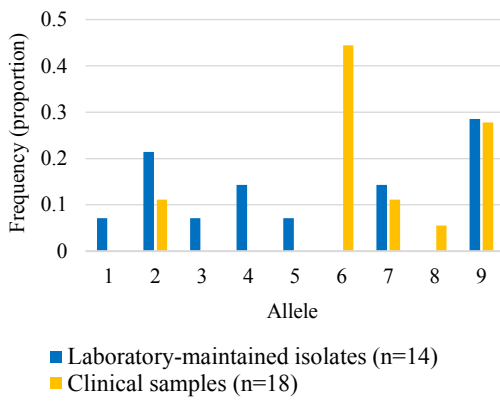
MRI_036



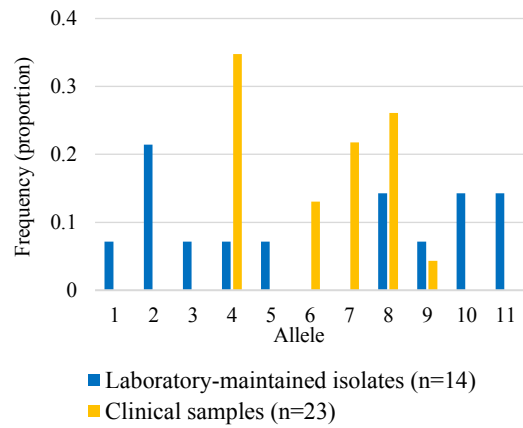
MRI_040



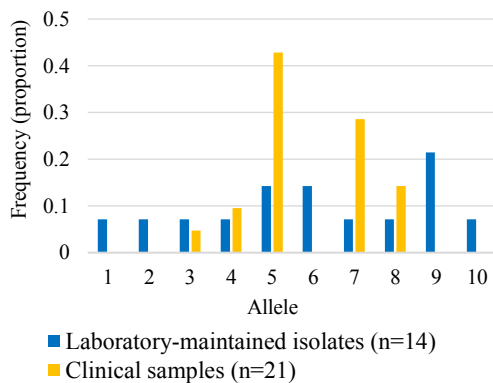
MRI_037



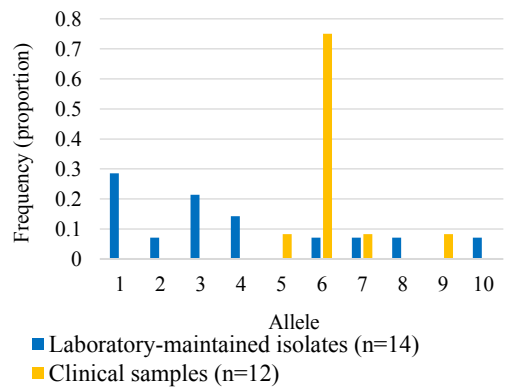
MRI_041



MRI_042



MRI_045



4.3.6 Cluster analysis

The relationship between laboratory-maintained isolates and clinical samples were investigated by constructing a neighbour-joining (NJ) dendrogram. This analysis was limited to those individual laboratory-maintained isolates ($n=14$) and clinical samples ($n=8$) for which an allele number was confidently allocated at all 12 microsatellite markers. Although the number of samples analysed was reduced, complete genotypic profiles were clustered using a distance-based phylogenetic algorithm. More refined genetic clustering should be applied to larger numbers of isolates to enable reliable inference on the population structure of the parasite. Nevertheless, this preliminary analysis provides evidence of sub-clustering of the samples obtained from abortion cases collected in the Dumfries and Galloway region of Scotland. Similarity between 3 samples showing identical MLG and 1 sample collected from the same herd were also pinpointed (Figure 4.4).

No obvious relationship amongst the other European, North American and Japanese isolates was observed depending on their geographical origin. However, the Japanese bovine isolates JAP-2 and JAP-4 formed a sub-cluster with the BPA-1 isolate from the United States (Figure 4.4).

4.3.6.1 Polymorphism of microsatellite markers in Scottish clinical samples in relationship to herd of origin and abortion outbreak

Within the Scottish samples, the analysis of complete and partial MLGs, obtained from samples collected from the same herd, provided preliminary information on the distribution of different genotypes at the herd level in relationship to specific abortion outbreaks. All foetuses analysed were aborted by different dams.

In herd 5, the analysis of the 11 samples available showed that only the microsatellite markers MRI_014, MRI_030 and MRI_037 were polymorphic. The initial *N. caninum* abortion outbreak occurred in March 2010 was associated with the presence of a unique genotype (MLG 16). The full MLG was obtained for only 3 DNA samples. Partial multilocus profiles produced for another 3 samples were identical to MLG 16. Interestingly, a second abortion event that occurred on the same farm the following year in June was characterised by the presence of two different MLGs: the MLG 17 and an additional one showing different alleles at level of the MRI_030 and MRI_037

loci (Table 4.6). Two clinical samples were analysed from each of the herds 2, 8, 9, 10 and 11. At least 3 microsatellite markers were polymorphic in herd 2 (MRI_007, MRI_014 and MRI_041), 4 microsatellites in herd 8 (MRI_002, MRI_007, MRI_016, MRI_027) and 1 in herd 11 (MRI_027). In herds 2 and 8 both clinical samples originated from the same abortion outbreak: September 2009 (herd 2) and December 2008 (herd 8) respectively. In contrast, the clinical samples from herd 11 were collected following two distant abortion events in June and December 2008 (Table 4.6).

The partial MLGs obtained for the two clinical samples originating from herd 9 showed that none of the microsatellite markers, for which data were available, were polymorphic (Table 4.6). This may suggest that the two *N. caninum* DNA samples isolated following the same abortion outbreak may share an identical, or very similar, MLG. However, the unavailability of the full MLG data hindered definitive conclusions. Similarly, no polymorphisms were detected at any microsatellite markers, for which data were available, between the two clinical samples obtained from herd 10. These two DNA samples originated from two separate abortion outbreaks which occurred in March and September 2009 respectively (Table 4.6).

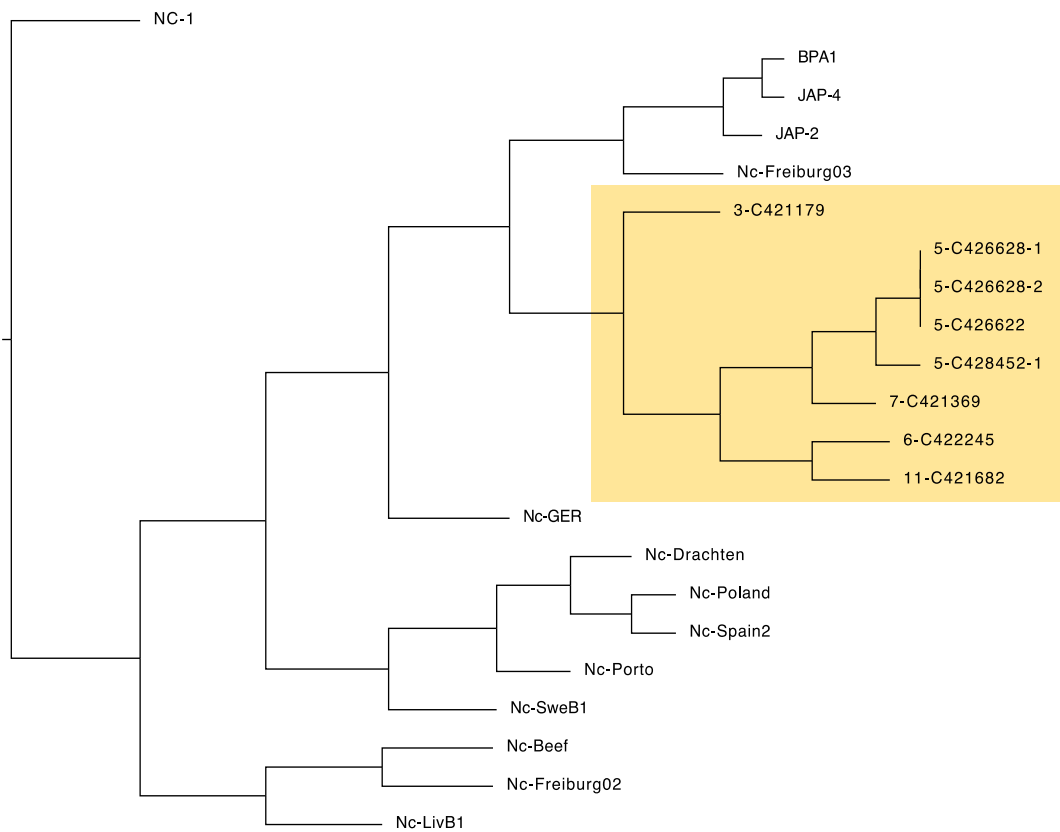


Figure 4.4 – Neighbour-joining (NJ) dendrogram showing the genetic relationships of laboratory-maintained and field *N. caninum* isolates based on multilocus analysis of the 12 polymorphic microsatellite markers investigated. Only the isolates in which an allele number was attributed in each of the 12 *loci* were included in the analysis. Clinical samples are shaded in yellow.

4.4 Discussion

4.4.1 Novel microsatellite markers for the genotyping of *N. caninum*

Discriminatory markers are a prerequisite for investigating the genetic diversity, taxonomy, epidemiology and pathogenicity of different *N. caninum* isolates (Regidor-Cerrillo *et al.*, 2006).

Microsatellite markers have been successfully applied to the study of genetic diversity of several apicomplexan parasites of human and/or veterinary interest including *T. gondii* (Ajzenberg *et al.*, 2010, Blackston *et al.*, 2001), *C. parvum* (Feng *et al.*, 2000, Hotchkiss *et al.*, 2015, Widmer *et al.*, 2004), *Theileria parva* (Katzner *et al.*, 2011, Oura *et al.*, 2003), *Plasmodium falciparum* (Anderson *et al.*, 2000b) and *Plasmodium vivax* (Karunaweera *et al.*, 2008) in addition to *N. caninum* (Al-Qassab *et al.*, 2009, Regidor-Cerrillo *et al.*, 2006).

Due to the high-resolution potential, the multilocus analysis of microsatellite markers has emerged as the gold standard technique for the differentiation of *N. caninum* at the strain or isolate level (Basso *et al.*, 2010). In addition, genotyping methods based on microsatellite markers can be applied on DNA samples obtained directly from infected tissues (e.g. tissues samples from aborted fetuses or afterbirths) (Pedraza-Diaz *et al.*, 2009) or parasite oocysts in canine faeces (Basso *et al.*, 2009) thus facilitating the genetic analyses of *N. caninum* for which the isolation of viable parasites is relatively difficult.

Most studies published to date have investigated the genetic heterogeneity of *N. caninum* using the panel of polymorphic microsatellite markers proposed by Regidor-Cerrillo and colleagues (2006). These microsatellite markers have been employed for the study of both laboratory-maintained *N. caninum* isolates, oocysts obtained from infected dogs and clinical samples from bovine and zebuine abortions (Basso *et al.*, 2009, Basso *et al.*, 2010, Brom *et al.*, 2014, Pedraza-Diaz *et al.*, 2009). Furthermore, based on these microsatellite *loci*, one large-scale study on the population structure of the parasite was carried out (Regidor-Cerrillo *et al.*, 2013). Additional polymorphic microsatellite and minisatellite markers were also reported and characterised by Al-Qassab and co-workers (2009).

The aim of the present work was to characterise further *N. caninum* microsatellite *loci* that can be used for genotyping this ubiquitous parasite and that can be applied on tissue samples collected from bovine aborted foetuses.

Twelve novel *N. caninum* microsatellite markers, that were not previously described, were investigated. *Locus*-specific nPCRs were developed and optimised using DNA samples isolated from *in vitro* cultured tachyzoites of different *N. caninum* isolates. Subsequently, the nPCRs were applied to the analysis of DNA samples extracted from infected foetal bovine tissues.

All investigated *loci* show extensive polymorphism thus were promising markers for the study of the parasite's genetic diversity.

Besides confirming that the fragment length polymorphisms were ascribable to the variations in the number of the repeat elements, sequencing revealed that many of the *loci* analysed did not contain simple tandemly repeated units but showed multiple variable repeat elements (Table 4.4). Markers containing a single variable repeat element, such as MRI_002, MRI_027, MRI_030, MRI_036, MRI_037 and MRI_040, are preferable as allele assignment can be confidently achieved based only on fragment size. In contrast, the combination of multiple repeated elements within the same *locus*, observed in the MRI_007, MRI_014, MRI_016, MRI_042 and MRI_045, would hamper the accurate allele assignment if the analysis is carried out exclusively by fragment sizing (Nadon *et al.*, 2013). Length variations occurring in more than one repeat within the same *locus* can result in alleles characterised by the same size despite showing different nucleotide sequences. For example, additions in a repeated element may compensate for deletions in another one without any gain or loss of nucleotides. In these cases, the exclusive use of automated fragment sizing would not detect sequence changes. As previously recommended for the well-characterised microsatellites MS2 and MS10 (Regidor-Cerrillo *et al.*, 2006), which are also characterised by multiple repeated elements, complex markers should be analysed exclusively by sequencing (Basso *et al.*, 2009).

Despite having a single polymorphic repeat, the marker MRI_041 described in this study was characterised by an AT motif in which substitutions of the T with a C occurred; therefore, this marker should also rely on sequencing analysis for accurate allele assignment.

Fragment size analysis by CE is generally cheaper than sequencing; consequently, it may be relevant to consider this aspect when validating panels of microsatellite markers to be applied on numerous samples. Preferably, microsatellite markers with simple motifs should be selected.

Most of the microsatellite markers used in this study had at least one polymorphic AT or TA repeat in their sequences. The abundance of AT tandem repeats in *N. caninum* was reported in a previous study (Regidor-Cerrillo *et al.*, 2006) and is consistent with other phylogenetically related apicomplexan parasites such as *T. gondii* (Ajzenberg *et al.*, 2002) and *C. parvum* (Feng *et al.*, 2000).

Although the nPCRs developed could amplify all 12 microsatellite markers from the DNA samples obtained from parasite isolates maintained *in vitro*, amplification was not achieved in all samples collected from bovine abortion cases in which the presence of *N. caninum* DNA was previously confirmed by ITS1 nPCR.

The proportion of ITS1 nPCR-positive clinical samples amplified by each microsatellite-specific nPCR ranged from 96.4% (95%CI, 82.3-99.4) to 43.0% (95%CI, 26.5%-60.9%) (Table 4.7). Such differences in the sensitivity of each nPCR protocol, that affected the typeability of each marker, may have been influenced by the PCR conditions applied. However, the quality of the DNA specimens that originated from foetal tissues which may have undergone autolysis or mummification, with consequent DNA degradation, may have also hindered amplification observed for some target *loci* in several samples. In addition, since the clinical samples were collected several years before this study, long-term storage may have influenced the DNA quality. Effectively, the nPCR protocols enabled the amplification of all laboratory-maintained *N. caninum* DNA samples that were isolated from tachyzoites cultured *in vitro* not long before testing.

Further studies using DNA samples recently extracted from non-autolytic aborted foetuses may help to clarify whether the quality of the DNA used might have had an effect on the low typeability observed for some microsatellite markers when applied to the clinical samples. Overall, the low typeability of some markers resulted in the successful assignment of a MLG in only 8 out of the 28 clinical samples examined (28.6%, 95%CI: 15.2%-47.0%). The nature of the sequences targeted by each microsatellite-specific nPCRs may have also had an impact on the sensitivity. Since

the ITS1 is a high-copy number element of the *N. caninum* genome (Homan *et al.*, 1997), DNA degradation may have had a less significant impact on the amplification of this target region when compared to the microsatellite *loci*. Reduced sensitivity of microsatellite nPCRs compared to the ITS1 nPCR was also observed in previous studies in which different markers were used (Pedraza-Diaz *et al.*, 2009, Regidor-Cerrillo *et al.*, 2013). Regidor-Cerrillo *et al.* (2013) reported that about 50% of the ITS1 nPCR positive samples produced complete or partially complete multilocus microsatellite profiles. In the present study, complete MLGs based on the full panel of markers were obtained only for 28.6% of the clinical samples (8/28, 95%CI: 15.2%-47.0%).

The analysis of laboratory-maintained isolates evidenced the high discriminatory ability ($SID \geq 0.75$) of the twelve markers described (Table 4.7). Within the clinical samples collected from a specific area of Scotland, the novel alleles detected at 8 out of 12 microsatellite *loci* (MRI_002, MRI_014, MRI_030, MRI_036, MRI_037, MRI_040, MRI_041, MRI_045) provided additional evidence of the discriminatory power of the markers (Figure 4.3). Interestingly, at the level of the MRI_036 *locus*, all clinical samples shared a unique, not previously observed, allele. The lower SID values obtained when considering the clinical samples independently or the totality of the samples analysed should be interpreted carefully. Some markers, that otherwise showed good discriminatory ability, displayed low SID depending on the fact that they were monoallelic (e.g. MRI_036) or not very polymorphic (e.g. MRI_016 and MRI_027) in the Scottish samples (Table 4.7). Furthermore, the unequal sample size resulting from the variable typeability of the markers should be considered.

The *locus*-specific nPCRs were unable to amplify DNA isolated from *H. heydorni* (isolate Giessen-1999), *T. gondii* (RH strain), *S. cruzi*, and *B. besnoiti* (Evora strain) suggesting the species-specificity of the molecular markers described in this study. A previous study by Regidor-Cerrillo and co-workers (2006) reported the amplification of 13 *N. caninum* microsatellite *loci* (MS1A, MS1B, MS2, MS3, MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, MS21) from the *H. heydorni* isolate Berlin-1996 with 7 markers showing unique alleles; however, morphological and genetic analysis proved that this isolate and *N. caninum* were indistinguishable (Regidor-Cerrillo *et al.*, 2006). Testing the 12 novel microsatellite-specific nPCRs on additional DNA samples

from different *H. heydorni* isolates is required to confirm the non-amplification of DNA from this apicomplexan species as observed for the isolate Giessen-1999 used in this study.

The *in vitro* stability represents another important feature that microsatellite markers should possess (Nadon *et al.*, 2013, Regidor-Cerrillo *et al.*, 2013). The reference, laboratory-maintained, *N. caninum* isolates are stored for prolonged periods of time and rapidly proliferating tachyzoites are periodically subjected to *in vitro* passages (Dubey *et al.*, 2007). Further investigations aimed at genotyping numerous isolates, generated in different laboratories through serial passaging of the same strain, are required to evaluate the influence of multiple passages on the conservation of the repetitive motif for each microsatellite *locus*.

This would help to exclude the occurrence of target *loci* modifications *in vitro*. Additionally, useful information on the stability of specific microsatellites may be obtained by genotyping attenuated and non-attenuated parasite strains kept *in vitro* for different lengths of time.

At the inter-laboratory level, it would be advantageous to agree on the genotypic characteristics of the reference laboratory-maintained strains of *N. caninum* by using a shared genotyping framework. This would ensure that identical parasite strains are used when *in vitro* or *in vivo* experiments are conducted by different research groups thus enabling meaningful comparison and validation.

4.4.2 Genetic diversity of *N. caninum* determined by multilocus microsatellite analysis

The MLG analysis revealed extensive genetic and genotypic diversity amongst 14 laboratory-maintained isolates of *N. caninum*. These isolates were obtained from both bovine and canine infected tissues as well as oocysts isolated from dog faeces collected in several different European countries, the United States of America and Japan (Table 4.1). Due to the diverse origin of these isolates, a degree of genetic polymorphism was expected.

The considerable genetic diversity of the parasite was also demonstrated by the differences in the frequencies of the predominant alleles between the reference (laboratory-maintained isolates) and the study population (clinical samples from a

specific area of Scotland) as well as by the detection of unique alleles in each population.

Such high genetic diversity was also observed when a smaller panel of 6 arbitrarily selected microsatellite markers was applied (Table 4.9).

These findings are consistent with previously reported microsatellite analyses of *N. caninum* (Regidor-Cerrillo *et al.*, 2013, Regidor-Cerrillo *et al.*, 2006). Likewise, considerable genetic diversity determined by multilocus microsatellite analysis was reported in the phylogenetically related apicomplexan *T. gondii* (Mercier *et al.*, 2011, Verma *et al.*, 2015).

Unsurprisingly and as indicated by the genetic and genotypic diversity indexes calculated for the two populations (Table 4.9), lower genetic diversity was observed amongst the clinical samples which were obtained exclusively from bovine aborted foetuses or placentas that were collected in herds located in a relatively restricted area of Scotland. Nevertheless, the detection of identical MLGs exclusively within individual herds suggested that *N. caninum* is a highly genetically diverse parasite also at the regional level. Similar observations were made within previous microsatellite analyses of *N. caninum* DNA samples following abortion outbreaks (Basso *et al.*, 2010, Pedraza-Diaz *et al.*, 2009, Regidor-Cerrillo *et al.*, 2013).

Clear sub-clustering of the clinical Scottish samples was observed (Figure 4.4); however, there was no obvious relationship amongst laboratory-maintained isolates depending on their geographical origin.

A level of geographical sub-structuring between *N. caninum* populations was previously observed through the MLFT analysis of parasite isolates originating from Spain, Argentina, Germany and Scotland (Regidor-Cerrillo *et al.*, 2013) (Figure 4.5). The closest relationships were between the Spanish and Argentinean populations, and between the German and Scottish populations (Regidor-Cerrillo *et al.*, 2013). A degree of sub-clustering of the Scottish DNA isolates was also reported (Figure 4.5). In the present study, the NJ analysis showed indications of a closer relationship between the population from a restricted area of Scotland and two laboratory-maintained *N. caninum* isolates originally from Germany (NcGer1, NcFreiburg03) (Figure 4.4). However, in the study of Regidor-Cerrillo *et al.* (2013), the same NcGer1 isolate did

not exhibit an apparent relationship with the Scottish isolates, nor with other German isolates (Figure 4.5).

The comparison between the two studies is difficult because the panels of microsatellite markers and the *N. caninum* isolates were both different. Importantly, the exclusive use of NJ analysis may fail to represent the complex population of *N. caninum* and accurately detect the relationships between geographical origin and genetic divergence (Pritchard *et al.*, 2000). Multivariate analyses, such as principal component analysis (PCA), have been employed extensively to efficiently extract biological information from genetic markers (Cavalli-Sforza, 1966). PCA reduces data by geometrically projecting them onto lower dimensions, designated principal components (PCs), with the aim to find the best summary of the data using a limited number of PCs (Lever *et al.*, 2017). Within the analysis of genetic markers, ordinations in reduced space are employed to find a few PCs that reflect as much of the genetic variability as possible (Jombart *et al.*, 2009). In the present study PCA was not performed because of the small sample size.

sources. Associated with the assessment of the abortion pattern (i.e. epidemic, endemic, sporadic) this information may help to confirm the predominant route of transmission hence shape appropriate control measures and prevention strategies.

Furthermore, genotyping *N. caninum* DNA samples from tissues of other domestic and wildlife intermediate host species, in which clinical disease is not observed, may enable clarification of their role as potential reservoirs. For example, identities between the MLGs of *N. caninum* DNA samples from wildlife and cattle co-habiting the same environment, in which definitive canid hosts are present, may provide additional information on the contribution of certain species to the epidemiology of neosporosis in specific farms or geographical areas.

Fragment size analysis by CE was chosen since it is less expensive than sequencing and therefore ideal for high-throughput analyses. Future studies may capitalise on the most recent high-throughput sequencing technologies (Reuter *et al.*, 2015) to develop cost-effective *N. caninum* genotyping methods based on multilocus sequence typing (MLST). MLST tools have shown comparable typeability but greater discriminatory power, accuracy and reproducibility than MLFT when applied to the genotyping of the protozoan parasite *C. parvum* (Diaz *et al.*, 2015). However, MLFT methods have the advantage of being able to readily detect mixed infections (i.e. different genotypes of the parasite in the same sample), which are identified by secondary peaks in the CE electrophoretograms (Hotchkiss *et al.*, 2016).

Whole genome sequencing might represent an alternative option for the study of the genetic diversity of *N. caninum*; however, its cost-efficiency might be limited.

Finally, the availability of additional whole genome sequences of different *N. caninum* isolates would have helped the *in silico* identification of optimal microsatellite markers characterised by conserved primer binding sites. This strategy has been used successfully to optimise the MLFT analysis of *C. parvum* by using the available sequenced genomes of several isolates (Perez-Cordon *et al.*, 2016).

4.5 Conclusions

The novel microsatellite markers described and evaluated in the present study were highly polymorphic and showed their potential applicability to the study of the genetic diversity of *N. caninum*. Importantly, the microsatellite amplification and fragment analysis protocols developed were successfully applied to DNA samples obtained from bovine aborted fetuses and placentas in which the parasite was previously detected.

The MLG offers a very useful discrimination typing tool although the analysis is highly dependent on the resolution of the markers used and sampling strategies applied. Further investigations, including the analysis of a larger number of laboratory-maintained, clinical and non-clinical (e.g. domestic and wild asymptomatic intermediate hosts) samples, are required to enable the selection of optimal markers that may contribute, in association with previously described microsatellites (Regidor-Cerrillo *et al.*, 2006, Al-Qassab *et al.*, 2010), to the generation of a standardised MLG framework that can be used at the inter-laboratory level as previously described for other apicomplexans of human and veterinary importance such as *C. parvum* (Chalmers *et al.*, 2017, Hotchkiss *et al.*, 2015).

Refined MLG methods based on microsatellite markers would be beneficial to investigate the source of infection and infection dynamics at the herd or regional levels. In addition, they would aid the study of the association between the genetic heterogeneity of *N. caninum* and the diverse biological features *in vitro* and *in vivo* (i.e. virulence and pathogenicity) that are seen amongst different isolates.

An international workshop to define and universally approve the number and choice of microsatellite markers to be used for genotyping *N. caninum* as well as determine the genotypic characteristic of the *N. caninum* laboratory-maintained isolates commonly used in research would be highly advantageous.

Chapter 5: Attitudes and perceptions of large animal veterinarians towards the diagnosis and control of bovine neosporosis

5.1 Introduction

The clinical advisory and consulting role of livestock veterinarians requires provision of advice to farmers and owners of the animals on the most appropriate herd health management practice for the local and national contexts (Caceres 2012). This implies a close collaboration with other stakeholders such as consultants of governmental animal health agencies, veterinary pathologists, clinical pathologists and specialists in the laboratory diagnosis of diseases as well as scientists involved in relevant research fields.

Bovine neosporosis is a ubiquitous cause of abortion, stillbirth and, less frequently, neonatal mortality. Cattle veterinary practitioners are asked to advise on the management of the disease on a regular basis (Haddad *et al.*, 2005). In this context, applying suitable diagnostic strategies and advising on the implementation of effective control measures are key areas in which appropriate veterinary input is most effective in helping to prevent and control the disease (Bronner *et al.*, 2014).

Abortion is the major problem associated with *N. caninum* infection and is seen most frequently between 4 and 7 months of gestation. Epidemic, endemic or sporadic abortion patterns are possible depending on the predominant transmission route (Goodswen *et al.*, 2013). The aborted fetuses are typically autolysed without any characteristic gross lesions and the aborting dams generally do not show clinical signs. Retention of the foetal membranes and metritis may represent secondary complications that follow abortion (McAllister 2016).

Frequently, *N. caninum* infections are subclinical; however, seropositive dams have an increased relative risk of abortion compared to seronegative. Meta-analysis of several different studies estimated that seropositive dams are approximately 3.5 times more likely to abort than seronegative (Reichel *et al.*, 2013).

Post-mortem, the presence of *N. caninum* is demonstrated by histopathological examination, immunohistochemistry, foetal serology, PCR and, rarely, isolation of the

parasite (Dubey and Schares 2006, Ortega-Mora *et al.*, 2006). In live animals, serology is the diagnostic tool of choice and is predominantly carried out using ELISA techniques although IFAT, agglutination tests, immunoblots and immunochromatographic methods are also available (Ghalmi *et al.*, 2014).

Herd-based management to reduce the economic and welfare costs of *N. caninum* at the herd level depend on the country or region, typology of cattle herd (e.g. dairy or beef suckler herds), infection rate and the associated risk factors which may vary considerably from farm to farm (Dubey *et al.*, 2017). At present, control options are restricted to management-based strategies relying on diagnosis of infection, informed breeding strategies and application of biosecurity measures due the lack of licensed vaccination and antiprotozoal treatments (Reichel *et al.*, 2014).

In the United Kingdom, several cattle herd health scheme providers offer a scheme for bovine neosporosis which is licensed by the Cattle Health Certification Standards UK since 2015 (CHeCS). The current scheme includes three programmes aimed at achieving accreditation as *N. caninum*-free herd, the eradication of the disease or the cost-effective control of bovine neosporosis at the herd level. All programmes are based on the identification of infected animals by serology and their removal from the herd (eradication programme) or exclusion from breeding replacement stock (disease control programme) (CHeCS 2016). In addition, biosecurity guidelines to prevent the reintroduction of the disease in uninfected herds as well as good practice recommendations for dealing with abortion cases are provided. Nevertheless, few farms across the country are currently enrolled in the scheme (CHeCS *Neospora* subgroup, personal communication).

In general, there are numerous temporal, cultural and social elements which play a role in the management of infectious disease outbreaks (Kahan *et al.*, 2010). This is also applicable to herd health issues, such as bovine neosporosis, in which the decisions taken by each veterinary practitioner are influenced by a multitude of individual (e.g. personal knowledge and experience) and social factors (e.g. attitude of farmers and colleagues to the problem). Nonetheless, the analysis of perceptions, attitudes, preferences and actions commonly taken to tackle *N. caninum* in cattle may help to better understand common veterinary practice in this specific context and identify current needs, knowledge gaps and potential areas of improvement.

In this chapter, a questionnaire-based survey for veterinary practitioners operating in the cattle sector was undertaken with the following objectives:

1. Survey current perceptions of *N. caninum* and bovine neosporosis among practicing veterinarians
2. Assess common practice and attitudes towards the diagnosis of *N. caninum*
3. Evaluate actions and adopted strategies for the management of bovine neosporosis
4. Identify needs and areas which may require improvement and knowledge gaps to improve the diagnosis and management of bovine neosporosis.

5.2 Materials and methods

5.2.1 Questionnaire design

The questionnaire consisted of three sections aimed at capturing different aspects related to the diagnosis and management advice relating to bovine neosporosis in current veterinary practice: (1) general assessment and perceptions, (2) diagnosis of bovine neosporosis and (3) control strategies. Basic demographic information was collected although the respondents were invited to complete the questionnaire anonymously.

The survey consisted of 24 questions. Different types of question were included in the questionnaire. Most questions were based on a 4 or 5 point Likert-type scale; the respondents were either asked to state the level of agreement or disagreement with certain statements or to specify how frequently they were used to make determined recommendations, make specific decisions or adopt control measures. Open-ended questions were also included and used predominantly to explore common practice within the diagnosis of *N. caninum* and opinions regarding the disease and how to minimise its impact (Appendix III).

The questionnaire was pilot tested with 6 cattle veterinary clinicians including representatives of consultancy institutions and a clinical academic. Two questions were removed and four revised or reworded.

5.2.2 Target population and distribution

The target population consisted of cattle veterinary practitioners in private practice or employed by diagnostic and research laboratories or public consulting veterinary institutions (i.e. veterinary investigation officers) with experience in cattle practice in the United Kingdom. The participants were selected by convenience sampling (Dohoo 2003).

The online questionnaire was created using the BOS online surveys tool produced and managed by the University of Bristol (<https://www.onlinesurveys.ac.uk>).

An invitation letter including the link to the online questionnaire was distributed via email. Mailing lists of the members of the Moredun Foundation who were in veterinary

practice and users of the Dairy Herd Health and Productivity Service (Royal (Dick) School of Veterinary Studies, the University of Edinburgh) were used to disseminate the survey.

In addition, paper copies were circulated among cattle veterinary practitioners who visited the Moredun Foundation marquee during the Royal Highland Show held in Edinburgh in June 2016 and at the UK Beef Expo event held at Melton Mowbray in 2016. Paper questionnaires were completed at the event and returned immediately after completion. Distribution and publicity about the survey were also placed on the social networking sites of the Moredun Foundation (Twitter and LinkedIn). The data were gathered between January and June 2016. Due to the multimodal distribution of the questionnaire and the uncertainty on the number of veterinarians reached, the response rate could not be calculated.

5.2.3 Data analysis

Replies were logged onto a spreadsheet (Excel, Office 2016; Microsoft Corporation, USA). Data from unanswered questions or answered ambiguously were excluded from the analysis. Basic descriptive statistics were used to report the data collected. Each open-ended question was analysed by assessing the frequency with which each answer option recurred across all responses. Following a preliminary analysis, specific words or wording unambiguously related to a topic, option or area were identified and grouped. For example, when the participants were asked to state the diagnostic test or tests which were regarded as those providing the highest level of confidence for formulating a diagnosis of neosporosis in aborted fetuses, statements such as “*lesions in foetal brain*”, “*pathology with lesions suggestive of protozoal abortion*”, “*foetal histology*” or “*microscopic lesions in foetal tissues*” were grouped as “histopathology”.

Sample proportions of participants selecting multiple choice answers or addressing open-ended questions with a determined topic, area or option were reported associated with 95% confidence intervals calculated using the Epitools confidence limits for sample proportions tool (<http://epitools.ausvet.com.au/>).

5.3 Results

A total of 53 respondents completed the questionnaire. Most respondents were veterinary surgeons in private practice (84.9%), about two-thirds of whom worked within the dairy cattle sector either exclusively (34.0%) or predominantly (32.1%). Less than one in ten responders were exclusively (1.9%) or mainly (7.5%) beef cattle practitioners (Table 5.1). Over half of the respondents (52.8%) were between 24 and 35 years old, just over one third between 36 and 50 years of age (35.9%) and only 6 (11.3%) were older than 50. Compared to other macro areas of Great Britain, Southern England was the region from which the highest number of completed surveys was received (Table 5.1).

Table 5.1 – Primary professional activity, field within the cattle sector, age, years in practice and region of professional activity of the veterinarians completing the questionnaire ($n=53$).

	No. Respondents	% Respondents
Primary activity		
Veterinary surgeon in private practice	45	84.9
Diagnostics/research	5	9.4
Veterinary investigation officer/consultant	3	5.7
Field of activity within the cattle sector		
Dairy	13	24.5
Beef	1	1.9
Mixed	18	34.0
Mixed mainly dairy	17	32.1
Mixed mainly beef	4	7.5
Age		
24-35	28	52.8
36-50	19	35.8
51-65	5	9.4
Over 65	1	1.9
Years in veterinary practice		
Less than 5 years	12	22.6
5-10	12	22.6
11-20	21	39.6
21-30	5	9.4
30 or more	3	5.7
Practice region		
Southern England	24	45.3
Midlands	8	15.1
Wales	2	3.8
Northern England	11	20.8
Scotland	8	15.1

5.3.1 Section 1: General assessment and perceptions

Over 96% (51/53, 95%CI: 87.2-99.0) of the veterinary surgeons interviewed stated that *N. caninum* was one of the three most frequently diagnosed infectious causes of bovine abortion in their geographical area of professional activity. Of these, 66.7% (34/51, 53.0-78.0) indicated *N. caninum* as the most important infectious abortifacient diagnosed. Bovine viral diarrhoea virus (BVDV) was listed as the most frequently diagnosed pathogen by 22.6% (12/53, 13.4-35.5) of respondents followed by infectious bovine rhinotracheitis virus (IBRV, officially known as BoHV-1) and *Bacillus licheniformis* both were mentioned by 3.7% (2/53, 1.1-12.7) of the participants. Two respondents highlighted that the most common outcome within the aetiological diagnosis of bovine abortions was an inconclusive result (Figure 5.1).

Overall, mycotic infections were cited within the first three main diagnosed causes of abortion relatively frequently (24.5%, 14.9-37.6); however, none of the respondents mentioned fungi as the most commonly diagnosed infectious cause of foetopathy. Likewise, salmonellosis was listed by 22.6% (12/53, 13.4-35.5) of the veterinarians but indicated as the most commonly observed only by 1.8% (1/53, 0.3-9.9) (Figure 5.1).

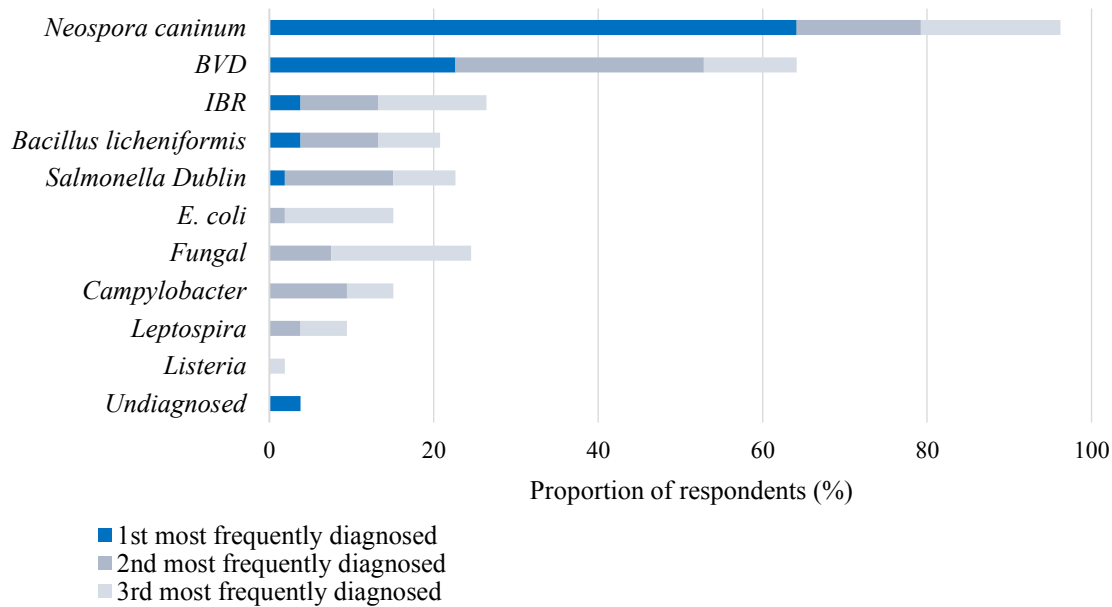


Figure 5.1 – Infectious causes of bovine abortion diagnosed most frequently according to the experience of the respondents in their geographical area of professional activity ($n=53$).

Among the causes of abortions cited, *N. caninum* infection was indicated as the most challenging to diagnose by 43.4% (23/53, 30.1-56.7) of the veterinarians followed by leptospirosis (13.2%, 6.5-24.8), which is primarily caused by *Leptospira borgpetersenii* serovar Hardjo and *Leptospira interrogans* serovar Hardjo in the United Kingdom, IBR (11.3%, 5.3-22.6) and BVD (9.4%, 4.1-20.2) (Figure 5.2).

Seven respondents (13.2%, 6.5-24.8) stated that all abortifacient infections were equally difficult to diagnose whilst two (3.7%, 1.1-12.7) specified that the greatest challenge was represented by those cases in which a conclusive diagnosis is not achieved. These responses were provided by the same veterinarians who highlighted the frequent occurrence of inconclusive diagnoses.

Most respondents indicated bovine neosporosis as the infection which poses the greatest challenges in terms of control (41/53, 64.5-86.5), whereas 13.2% (7/53, 6.5-24.8) and 3.7% (2/53, 1.1-12.7) considered respectively BVD and campylobacteriosis (*Campylobacter foetus* infection) as the most difficult infection to manage at the herd level; 5.6% (3/53, 1.9-15.3) said that all pathogens were uniformly difficult to manage (Figure 5.2).

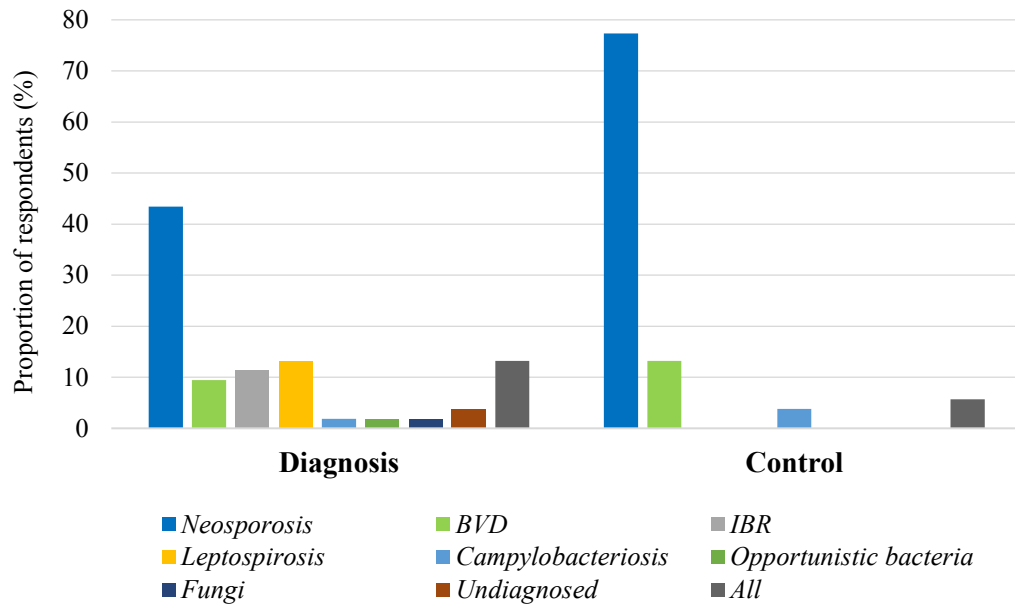


Figure 5.2 – Infectious causes of bovine abortion perceived as the most challenging in terms of diagnosis and control ($n=53$).

According to their personal experience, 62.3% (33/53, 48.8-74.1) and 3.7% (2/53, 1.0-12.7) of the respondents rated the economic impact of bovine neosporosis as high or very high respectively. The remaining 34.0% (18/53, 22.7-47.4) considered *N. caninum* as a cattle pathogen with low economic consequences. Of these, 3 (5.7%, 1.9-15.4) were beef cattle veterinary practitioners.

When questioned about their general views on the laboratory diagnosis of *N. caninum*, irrespective of the method used, about half of the participants (47.2%, 34.4-60.3) disagreed or strongly disagreed that in some cases they had doubts about the interpretation of diagnostic results, 39.6% (21/53, 27.6-53.1) agreed and the remaining 13.2% (7/53, 6.5-24.8) were unsure (Figure 5.3).

The clear majority of respondents agreed (69.8%, 56.4-80.4) or strongly agreed (17.0%, 9.2-29.2) that positive results obtained with current diagnostics were trustworthy. In contrast, most cattle practitioners either disagreed (41.5%, 29.3-54.9) or strongly disagreed (17.0%, 9.2-29.2) that negative diagnostic results were trustworthy. Just over one-third (34.0%, 22.7-47.4) of the participants were unsure about the reliability of negative diagnostic results (Figure 5.3).

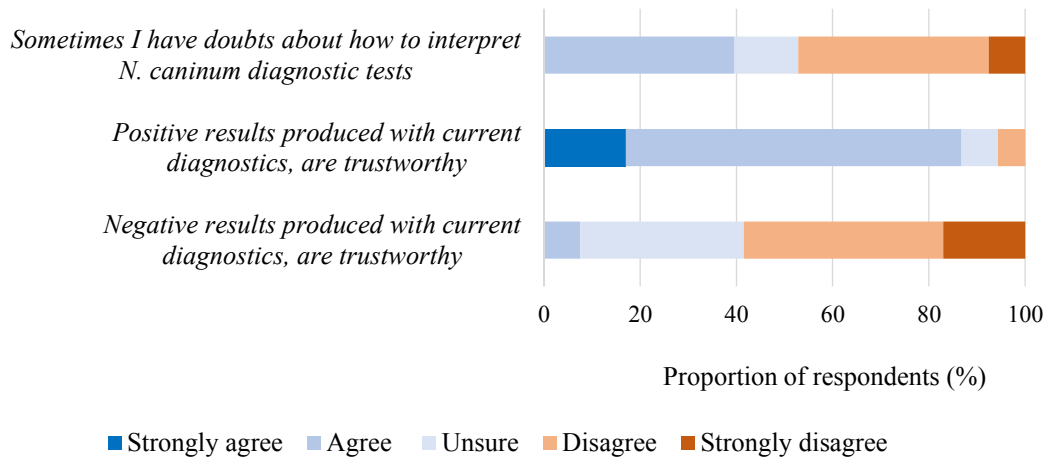


Figure 5.3 – General perceptions on the laboratory diagnosis of bovine neosporosis (n=53).

In general, the veterinarians interviewed felt confident about their understanding of the life cycle of *N. caninum* and bovine neosporosis; however, most of them (83.3%, 70.7-90.8) disagreed or strongly disagreed that farmers understood the cycle of the parasite (Figure 5.4). General agreement with statements covering the importance of controlling bovine neosporosis to improve the profitability of dairy and beef farms was observed. Nevertheless, about half (50.9%, 37.9-63.9) of the veterinarians disagreed that their clients perceived bovine neosporosis as a major threat to their farming businesses (Figure 5.4).

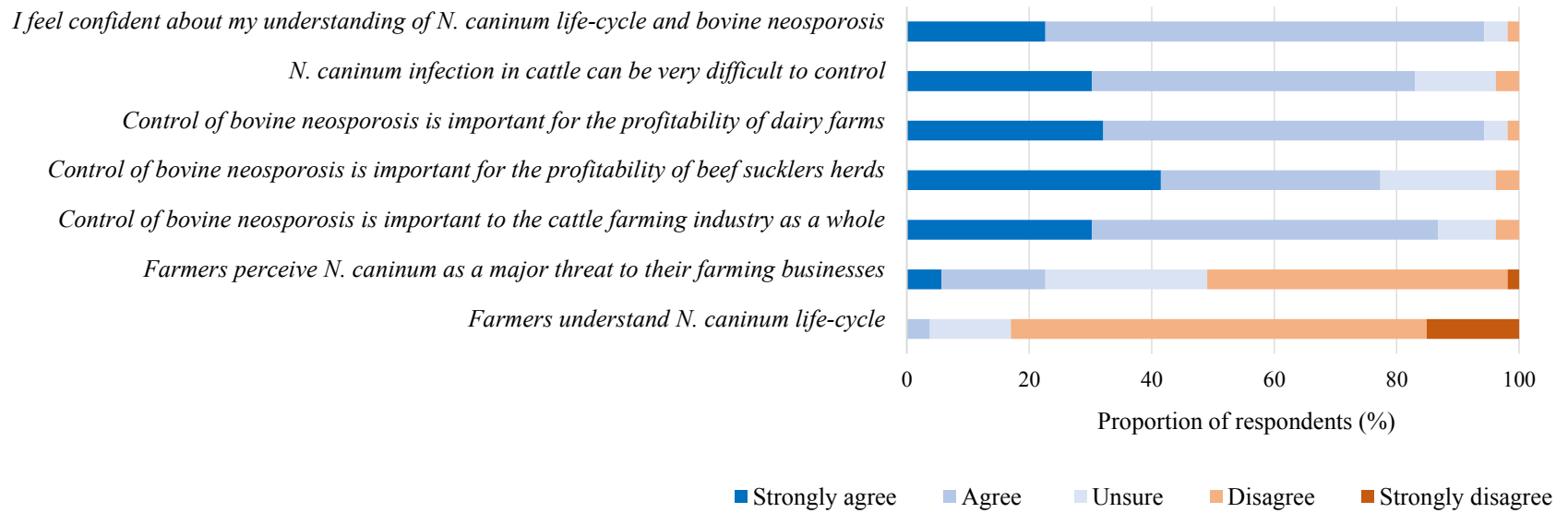


Figure 5.4 – General perceptions of *N. caninum* and bovine neosporosis ($n=53$).

5.3.2 Section 2: Diagnosis of bovine neosporosis

The association of *N. caninum* infection with one or more abortion cases, the occurrence of one abortion storm and the persistence of endemic abortions attributable to the protozoan were identified as the situations in which 88.7% (47/53, 95%CI: 77.4-94.7), 71.7% (38/53, 58.4-82.0) and 67.9 (36/53, 54.5-78.9) of the respondents respectively would start to be concerned about the presence of *N. caninum* in a herd. These were the main warning signs alerting cattle practitioners to initiate specific further testing for *N. caninum*. The occurrence of abortion storms on neighbouring farms was a concern for only 15.1% of respondents (8/53, 7.8-27.0) (Figure 5.5). Other indicators that may suggest the possible involvement of *N. caninum* to the respondents were: general poor fertility performance, difficulty to get heifers pregnant, the presence of young dogs on the farm, history of low incidence of abortion not previously investigated and the presence of a bulk milk positive result for the presence of *N. caninum* specific antibodies.

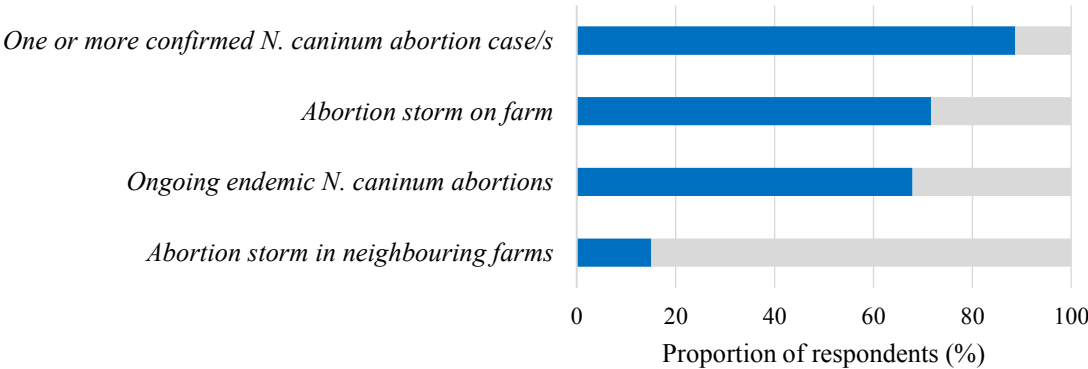


Figure 5.5 – Main triggers for the implementation of additional testing and *N. caninum*-specific control measures (n=53).

Understandably, 98.1% (52/53, 90.1-99.7) of the cattle veterinary practitioners interviewed said that their approach of choice in the case of suspected *N. caninum* abortion was the submission of the foetus to a veterinary investigation laboratory. Thirty-five percent of the respondents (19/53, 24.3-49.3) added that they would also submit a serum sample from the aborting dam for serological testing. Likewise,

advising farmers to submit aborted fetuses for histopathological examination and foetal serology as well as recommending the submission of serum samples from aborting dams were actions undertaken always by 60.3% (32/53, 49.9-72.2) and 66.0% (35/53, 53.6-77.3) of the respondents respectively. Such advice was frequently given by all other respondents except for one (Figure 5.7).

Within the laboratory investigation of abortion cases, the diagnostic strategy which was generally perceived to enable the diagnosis of neosporosis with the highest level of confidence was, according to 69.8% (37/53, 55.4-80.4) of the respondents, a combination of two or more methods. Two respondents explained that they considered as equally reliable any positive result regardless of the test or technique used and another 2 participants were unsure.

The histopathological examination of the aborted foetus associated with either the detection of *N. caninum* DNA in aborted material by PCR or the serological testing of the aborting dam were the most frequently mentioned combinations of diagnostic techniques both accounting for 20.4% (10/49, 11.5-33.6) of the answers received.

Overall, histopathology was the diagnostic method most frequently mentioned; 67.3% (33/49, 53.4-78.8) of the respondents referred to this technique. Of these, 12.1% (4/33, 4.8-27.3) specified that the use of immunohistochemistry to support histopathological findings increased their confidence in formulating a diagnosis of bovine neosporosis. PCR on foetal tissues was indicated by 42.9% (21/49, 30.0-56.7) of the respondents followed by maternal (38.8%, 26.4-52.7) and foetal serology (24.5%, 14.6-38.1). Maternal and foetal serology were always mentioned associated with at least one other diagnostic method (histopathology or PCR) and/or combined with each other. However, only 12.9% (4/31, 3.2-28.8) of the veterinarians who mentioned either maternal, foetal serology or both said that they felt confident to formulate a diagnosis of neosporosis based exclusively on the outcome of serological methods.

Maternal serology was commonly applied and regarded as a useful tool to support the diagnosis of *N. caninum* infection in aborting dams. However, two-thirds of the respondents (66.0%, 53.6-77.3) agreed or strongly agreed that, in some cases, aborting dams can test serologically negative despite direct evidence of *N. caninum* being found in the aborted foetus (Figure 5.6).

In non-aborting animals, the assessment of the *N. caninum* infection status (i.e. infected or uninfected) was performed by all veterinarians (100%, 93.2-100.0) through the individual serological testing of a sample of cows. Approximately 55.0% (29/53, 41.4-67.3) of the respondents said that they sampled animals in late pregnancy; of these, 31.0% (9/29, 17.3-49.3) mentioned the specific 12 to 4 weeks pre-calving sampling window which has been historically recommended and is still included in the standardised official herd health scheme for bovine neosporosis. The remaining participants tested pregnant cows irrespective of the gestational stage (18.9%, 10.6-31.7) or both pregnant and non-pregnant cows (26.4%, 16.4-39.6). Serological examination of pre-colostral calves was mentioned in association with pre-calving maternal testing by a minority of veterinarians (3.7%, 1.0-12.7). In addition, three respondents (5.7%, 1.9-15.4) said that they also considered testing neonatal calves within the first 6 months of life.

In dairy herds with a history of neosporosis, 30.2% (16/53, 19.5-43.5) of the respondents commonly tested bulk milk samples for *N. caninum*-specific antibodies to screen for the presence or absence of the parasite. These were all cattle practitioners whose clients were exclusively or predominantly dairy farmers and accounted for more than 50% of the respondents in this group (16/30, 36.1-68.0). Nevertheless, individual serology in pre-calving cows was considered as preferable to bulk milk testing by the clear majority of participants (86.8%, 75.1-93.4) who also acknowledged that a negative bulk milk result would not exclude the presence of the parasite in the herd (92.4%, 82.1-97.0) (Figure 5.6).

All participants were specifically asked whether they thought serology-based diagnostics for *N. caninum* (ELISA format) gave reliable information on the exposure of the animals to the parasite: 54.7% (29/53, 41.4-67.3) provided an affirmative response, 24.5% (13/53, 14.9-37.5) were unsure and 20.7% (11/53, 12.0-33.5) did not think that the information obtained using the serological test was reliable. The reasons given to support views on the reliability of serological tests are shown in Table 5.2. Most participants who regarded serological testing as reliable added that there are important considerations to make when interpreting test results. These included the fluctuations of *N. caninum*-specific antibody titres as well as the occurrence of false negative results which might be attributable to the fact that antibody titres fluctuate

but also to the characteristic of the serological tests currently available. The remaining participants rarely had doubts about positive test results and perceived the specificity of the serological methods available as satisfactory or highlighted the fact that there are no other options for the diagnosis *in vivo* (Table 5.2).

The respondents who thought that current serological diagnostics did not give reliable information mentioned the low sensitivity whereas two specified that, in their opinion, the antibody titre dynamics was at the origin of the unreliability of serological results.

Table 5.2 – Views on the reliability of current serology-based diagnostics for bovine neosporosis ($n=53$).

Do current <i>N. caninum</i> serology-based diagnostics (ELISA) give reliable information? Why or why not?		No. Respondents	% Respondents (95%CI)
	<i>“Important consideration must be made for correct interpretation of test results (i.e. antibody titres wax and wane, limitations of available tests)”</i>	16	30.2 (19.5-43.5)
	<i>“High specificity: positive results are trustworthy.”</i>	7	13.2 (19.5-43.5)
YES	<i>“It is all we have got.”</i>	4	7.5 (3.9-17.9)
	<i>“The results are consistent over time.”</i>	1	1.9 (0.3-9.4)
	<i>“The results generally fit the clinical picture.”</i>	1	1.9 (0.3-9.4)
		29	54.7 (41.4-67.3)
	<i>“Low sensitivity / uncertainty about negative results.”</i>	9	17.0 (6.5-24.8)
NO	<i>“Fluctuations of antibody titres.”</i>	2	3.8 (1.1-12.7)
		11	20.8 (12.0-33.5)
Unsure		13	24.5 (14.9-37.6)

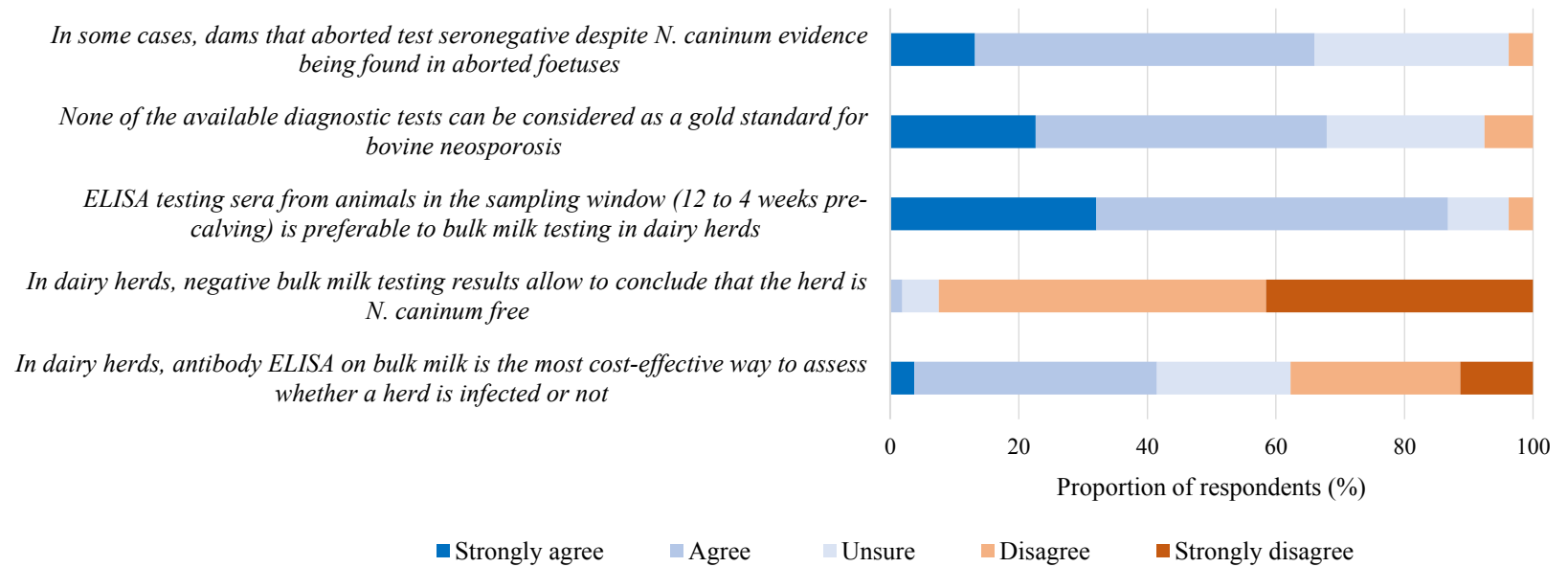


Figure 5.6 – Assessment of the presence or absence of *N. caninum* at the herd level ($n=53$)

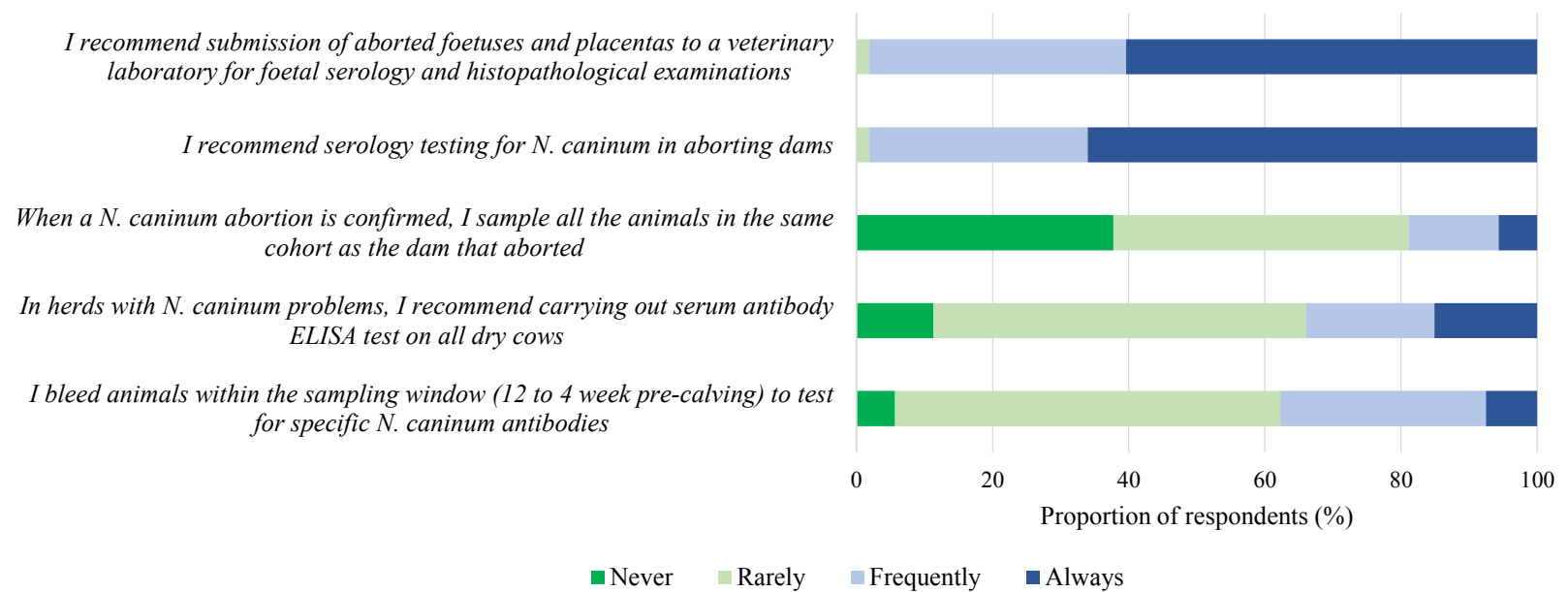


Figure 5.7 – Approaches taken during the investigation of *N. caninum* abortion outbreaks (n=53).

5.3.3 Section 3: Control strategies

Approximately 40% (22/53, 95%CI: 29.3-54.9) of all respondents said that their veterinary practice had a common policy and agreed guidelines for the advice given to farmers in the implementation of control programmes for bovine neosporosis. Where present, practice policies and guidelines were predominantly based on the information provided by one of the herd health schemes providers operating in the United Kingdom (63.5%, 42.9-80.3) and were applied regardless of whether each specific farm had joined a health scheme or not. All providers mentioned were CHeCS accredited.

Independently from the presence of defined practice policies, the control of the definitive canine host (i.e. farm and non-farm dogs) and the serological testing of the breeding stock were the key aspects emphasised by the veterinarians when advising farmers on the control of the disease. These aspects recurred in 54.7% (29/53, 41.4-67.3) and 52.8% (28/53, 39.6-65.6) of the answers analysed. Vertical transmission of the parasite and the measures aimed at preventing it, the thorough investigation of abortion cases and general biosecurity and hygiene measures (i.e. removal and appropriate disposal of aborted foetuses and afterbirths) were additional key points frequently emphasised.

High awareness and consideration of the importance of adequate biosecurity was observed. Collectively, over 90% of the veterinarians strongly agreed (45.3%, 32.7-58.5) or agreed (49.2%, 36.2-62.1) that herd biosecurity plans should include specific measures for the control of bovine neosporosis. At the same time, the clear majority of the respondents (88.7%, 77.4-94.7) acknowledged that the introduction of *N. caninum* in a herd occurred frequently despite the implementation of good biosecurity measures and almost 70% of them agreed (49.1%, 36.1-62.1) or agreed strongly (20.7%, 12.0-33.5) that newly introduced cows posed a high risk of introducing the parasite in uninfected herds (Figure 5.9). However, less than one-third of the veterinarians (30.2%, 19.5-43.4) said that they always or frequently recommended serological testing of prospective purchased heifers and cows (Figure 5.10).

The management of dogs' access to cattle and feed storage areas was at the centre of the biosecurity and general hygiene measures for the prevention of neosporosis. Nevertheless, only 13.2% (7/54, 6.5-24.8) of the respondents also considered testing

farm dogs for *N. caninum* prior to implementing control programmes for cattle. Of these, five stated that they would carry out serological testing in dogs whereas two would request direct detection of parasite's oocysts in faecal samples.

Increased public engagement to make dog owners aware of the potential risk of *N. caninum* transmission to cattle posed by their animals was considered necessary by the clear majority of the respondents (94.3%, 84.6-98.1) (Figure 5.8).

In terms of control strategies, about a quarter of the respondents (24.5%, 14.9-37.6) said that they would frequently consider a non-proactive "live with the disease" approach in herds in which the impact of neosporosis was perceived as low whereas 75.5% (40/53, 62.4-85.1) would rarely (42.3%) or never (30.2%) consider such attitude to the problem (Figure 5.9).

In a scenario characterised by a within-herd seroprevalence greater than 10% about one in ten veterinarians (88.6%, 77.4-94.7) would have frequently (41.5%) or always (47.1%) advised selective breeding of heifers which were born from seronegative dams. In herds with low seroprevalence (less than 5%) culling of seropositive cows would have been frequently or always advised by 64.1% (34/53, 50.6-75.7) of the respondents collectively.

Less than one-fifth (18.9%, 10.6-31.4) of the cattle practitioners interviewed always or frequently advised embryo transfer from infected donors to seronegative recipients as a measure to control *N. caninum* transmission in high-genetic merit herds; the majority would give such advise rarely (56.6%, 43.3-69.0) (Figure 5.9).

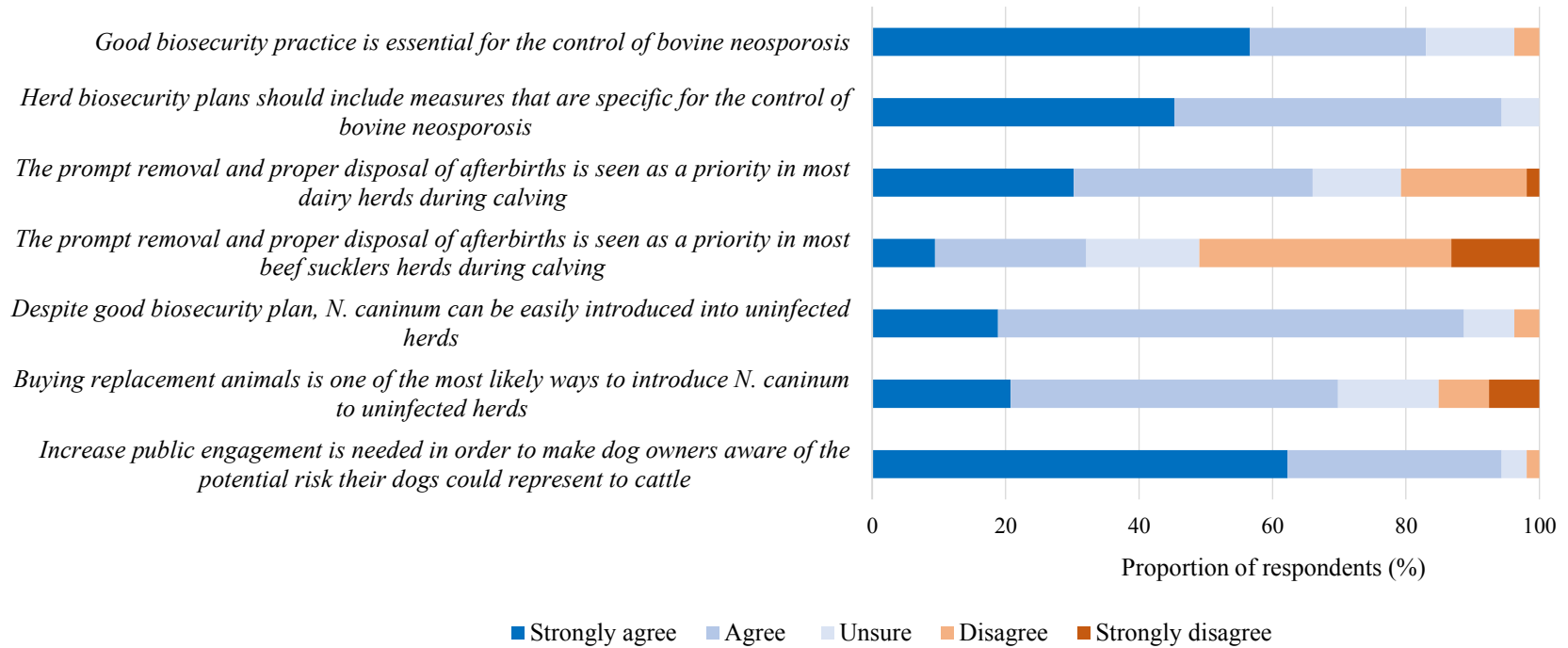


Figure 5.8 – Views on biosecurity and general hygienic measures ($n=53$)

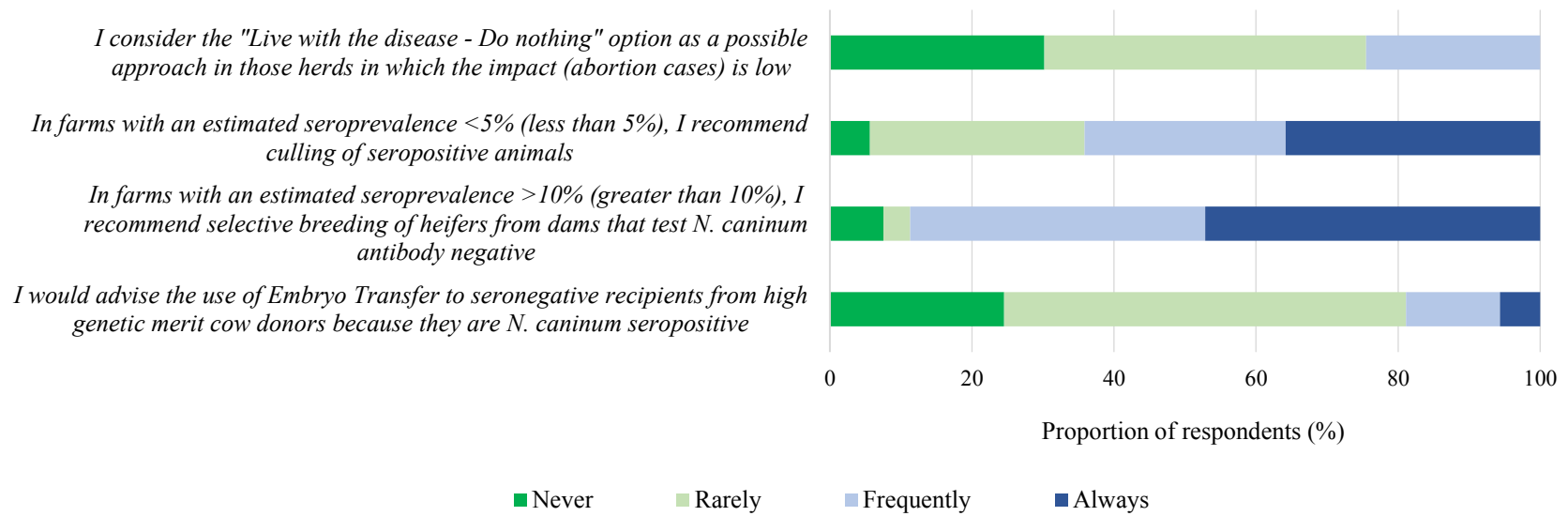


Figure 5.9 – Common practice and actions within the control of bovine neosporosis ($n=53$).

One third of the respondents (33.9%, 22.7-47.4) indicated the unavailability of a vaccine as one of the major limitations to the control of bovine neosporosis. This was followed by the farmers' attitude to the problem and the limitations of current diagnostic tests which were mentioned in 28.0% (15/53, 18.0-41.5) and 26.4% (14/53, 16.4-39.6) of the answers analysed respectively.

The farmers' attitude towards the problem (including low perception of the impact of neosporosis at the herd level, presence of other priorities or incomplete understanding of the disease) was the second most frequently mentioned limitation of control efforts. This can be summarised with the following statement made by one of the respondents: *“Lack of awareness of prevalence and financial implications of Neospora. This may be due to more pressing need of modern farmers as many diseases are perceived to be, and indeed may be, more damaging to profitability than neosporosis (e.g. mastitis and lameness).”*

The limitations of current diagnostic tests all referred to the lack of sensitivity of the serological methods and difficult diagnosis *in vivo* to identify persistently infected animals.

Other limitations highlighted were related to the intrinsic characteristics of *N. caninum* and its life-cycle (i.e. possibility of vertical transmission without overt clinical signs in either the dam or the offspring and establishment of persistent infections), cost of testing, difficult control of non-farm dogs' access to cattle housing and grazing areas (footpaths, dog walking areas) and the complicated identification of the source of infection which appeared in 18.9% (10/53, 10.6-31.7%), 13.2% (7/53, 6.6-24.8), 11.3% (6/53, 5.3-22.5) and 5.7% (3/53, 1.9-15.3) of the answers respectively.

When asked their views about prospective tools to control abortions due to neosporosis over three-quarters (84.9%, 72.9-92.9) of the veterinarians interviewed declared that they would always or frequently consider the use of vaccination if available. In contrast, 37.7% (20/53, 25.9-51.2) of the respondents would always or frequently recommend the administration of antimicrobials if these were licensed for the prevention of *N. caninum* abortions (Figure 5.10). Almost all respondents (96.2%, 87.2-99.0) strongly agreed or agreed that bovine neosporosis should be included in herd health schemes whereas the remaining two individuals were unsure (3.8%, 1.1-

12.7%). However, less than a quarter (22.6%, 13.4-35.5%) reported that farmers did not perceive accreditation for bovine neosporosis as an added value to their businesses.

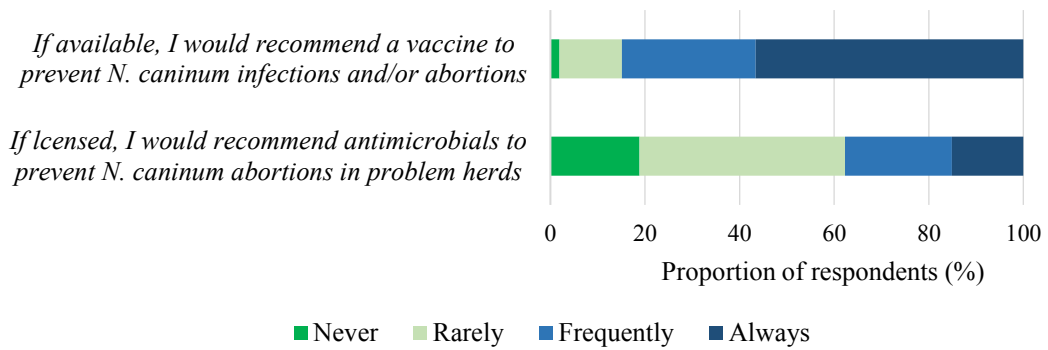


Figure 5.10 – Prospective use of vaccination and antimicrobial therapy for the control of *N. caninum* abortion ($n=53$).

5.4 Discussion

Consistent with the official data available for England, Scotland and Wales (VIDA 2015), *N. caninum* was regarded as the most frequently diagnosed infectious cause of abortion in cattle by the majority of veterinary practitioners interviewed. However, discrepancies between the perception of the respondents and the data available were observed for other abortifacients. For example, *Bacillus licheniformis*, the second most frequently diagnosed cause of bovine abortion in the United Kingdom (VIDA, 2015), was listed by the participants less frequently than BVD and IBR (Figure 5.1). According to the official data, these pathogens were ranked as the fifth and thirteenth abortifacient respectively (VIDA, 2015). The high overall impact of the two viral diseases in affected farms in terms of hypo- or infertility, in addition to abortion (Lanyon *et al.*, 2014, Nandi *et al.*, 2009), might have influenced the perceptions of the respondents. Importantly, the individual perception depends directly on the personal experience and the frequencies of certain bovine pathogens vary significantly depending on the region and specific area of the country.

Although the rate of definitive diagnoses formulated in the case of abortion was not a subject covered in the survey, two respondents highlighted that, in most cases, an aetiological diagnosis is not reached. Effectively, in 2015, the identification of the causative agent occurred in only 15.5% of the cases of foetopathy submitted to the veterinary investigation centres (VIDA 2015) thus highlighting an important problem faced by the veterinary practitioners during the investigation of cattle abortions.

N. caninum was considered the abortifacient of cattle most challenging to diagnose and control by most participants; however, a higher proportion (77.4%, 64.5-86.5) perceived the protozoan as particularly challenging to control rather than diagnose (43.4%, 30.1-56.7). Interestingly, leptospirosis which is normally considered difficult to control since it can be caused by several different serovars transmitted by various domestic and wild animals, was considered difficult to diagnose but not to control (Figure 5.2). Unlike neosporosis, leptospirosis can be controlled using vaccines against the bovine-adapted serovars as well as antimicrobials (Mughini-Gras *et al.*, 2014). The availability of these control tools may be the reason the disease was not listed amongst the abortifacient difficult to control.

Consensus on the importance of controlling *N. caninum* infection in both dairy and beef herds was observed indicating that the disease is regarded as an important health and welfare issue whose control would benefit individual farm businesses as well as the cattle industry as a whole (Figure 5.2).

Farmers' understanding of *N. caninum* life cycle was rated as insufficient by numerous respondents (83.3%) thus suggesting that enhanced knowledge transfer from veterinary practices as well as agricultural education institutions may be required. Alternatively, additional effort from the individual veterinarian may be necessary to inform those farmers who might not be up to date and guide them through the key aspects of the disease. A good understanding of the mechanisms and main routes of transmission would help farmers to implement control measures more proactively and effectively.

In spite of the fact that the economic impact of bovine neosporosis was rated as high or very high by almost two-thirds of the veterinarians (66.0%), approximately half of the respondents (50.9%) disagreed that farmers perceived the disease as a major threat to their businesses. A comparison between dairy and beef veterinary practitioners would have enabled the investigation of potential differences in approaching the disease between the two groups; however, this was not possible due to the low number of beef cattle veterinarians who undertook the survey (Table 5.1). The higher number of respondents involved in the dairy sector who completed the survey may suggest a higher interest of dairy veterinarians to the topic. However, the distribution method (i.e. use of the mailing list of the Dairy Herd Health and Productivity Service in addition to other distribution channels) was the likely cause of the "dairy bias" observed.

Interestingly, probing the general views about *N. caninum* and neosporosis provided an initial indication of the attitudes towards current *N. caninum* diagnostics. Regardless of the diagnostic method used, a different consideration on the reliability of positive and negative test results was observed. Over 75% (76.8%) of the participants said that they considered a positive result trustworthy whereas only 7.5% agreed that a negative result obtained with current diagnostics was trustworthy and 34.0% of the sample was unsure (Figure 5.3).

Besides being a legal requirement in the UK, the submission of aborted foetuses for further investigations enables a general diagnostic workup to be done to include a range of major cattle abortifacients rather than focusing on a specific one. This is regarded as the optimal way to approach an abortion problem at the herd level (McAllister 2016). In addition, the submission of serum samples from aborting dams for serology can provide additional diagnostic information during abortion outbreaks. Both these practices were well established and almost all (98.1%) of the participants stated that they frequently or always recommended such measures suggesting high consistency of action in this area (Figure 5.7).

The need for using different diagnostic techniques in parallel was a common notion amongst the veterinarians interviewed and 69.8% of them required a combination of two or more diagnostic methods for formulating a confident diagnosis of neosporosis. Effectively, several studies have illustrated this need by reporting limited agreement between histopathology, immunohistochemistry, PCR methods and foetal serology (Pereira-Bueno *et al.*, 2003, Sanchez *et al.*, 2009, Wouda *et al.*, 1997).

Histopathological examination and PCR detection of *N. caninum* DNA in tissues from aborted foetuses were the most frequently mentioned techniques for the investigation of abortion cases.

In non-aborting cattle, individual serology was regarded as the method of choice for determining the infection status of the animals. Testing cows in late pregnancy was relatively common and more than half of the veterinarians (54.7%) were used to testing cows during this reproductive stage. As discussed in Chapter 3 (3.4.2.1) screening cattle in mid to late pregnancy has been traditionally advised as it is thought to increase the chances of detecting specific *N. caninum* antibodies (Dannatt 1997).

The analysis of serum samples from pre-colostral calves paired to samples from their mothers is useful for the determination of the rate of vertical transmission and the predominant transmission route at the herd level (Dijkstra *et al.*, 2001a, Dubey *et al.*, 2007). However, at the time of the survey, this practice was not common as it was mentioned by only 3.7% respondents.

A limited proportion of respondents (5.7%) mentioned that they carried out serological testing in calves younger than 4 (1.9%) or 6 (3.8%) months. Unfortunately, the participants did not provide justification for this testing method which diverged from

the information available in literature. Since these responses were provided by a beef cattle veterinarian and two mixed beef and dairy cattle practitioners, it may be possible to speculate that the objective might have been to indirectly assess the serological status of the mother by detecting specific antibodies in post-colostral calves. If this was the case, the usefulness of this practice would be dubious.

Current serological methods, specifically those based on the ELISA format, were considered to provide trustworthy information about the *N. caninum* infectious status by 57.4% of the respondents. In contrast, 20.8% did not think that *N. caninum*-antibody ELISAs provided reliable information and 24.5% were unsure. Given the wide variety of diagnostic tests, both commercial and in-house, as well as the cut-offs used by different laboratories across the country, this outcome of the questionnaire should be interpreted as a general insight on the perceived reliability of serology-based diagnostics for *N. caninum* in cattle veterinary practice.

Interestingly, irrespective of whether these assays were deemed as trustworthy or not, the awareness of the suboptimal sensitivity of the tests, which was either attributed to the tests themselves or the fluctuation of antibody titres emerged in most answers.

Over 58% of the veterinary practitioners interviewed said that their practices did not have any agreed protocol for the control or management advice for *N. caninum* in infected herds. Although based on the same general principles, cost-effective strategies to control *N. caninum* are herd-specific and depend on the infection rate, associated risk factors, economic constraints and business objectives. Nevertheless, the use of protocols or guidelines agreed at the veterinary practice level may help to streamline the implementation of control and management measures, standardise the advice provided and, if reviewed regularly, include up to date scientific information according to the principle of evidence based veterinary medicine.

The assessment of *N. caninum* infection in farm dogs was considered and potentially carried out by a relatively small proportion (13.2%) of the respondents.

Dogs develop antibodies against *N. caninum* either post-natally, following the ingestion of food containing tissue cysts, or pre-natally, when foetuses are infected via the transplacental route (Dubey *et al.*, 2017). Retrospective testing of dogs naturally infected post-natally showed that *N. caninum*-specific antibodies can persist for up to 4 years; however, in animals with initial low titres (1:50), the antibody levels declined

below the detection limits of the tests used within a 1-2 year period (Barber and Trees, 1998). Besides the IFAT, which is the most commonly used test in dogs, various iELISAs and NATs have been described. These include several commercial assays for the detection of *N. caninum*-specific antibodies in cattle, which have also been validated to test canine sera (reviewed in Dubey *et al.*, 2017).

Serological testing would provide evidence that farm dogs have been exposed to the parasite; however, it is unclear whether previously exposed canids would have a reduced risk of shedding oocysts following repeated infections. On the other hand, direct detection of parasite oocysts in faecal samples may help to identify the source of infection during abortion outbreaks; however, by the time of testing, farm dogs may no longer be shedding oocysts. Although one study reported prolonged shedding in naturally infected dogs (McGarry *et al.*, 2003), there is evidence that experimentally infected canids only shed oocysts for about one week (Gondim *et al.*, 2002, Rodrigues *et al.*, 2004). Irrespective of the technique used and from a practical point of view the added value of testing farm dogs is questionable since there is no clear strategy about what to do with the information (McAllister 2016).

Cattle veterinary practitioners acknowledged the importance of including bovine neosporosis in herd health schemes; however, they also reported that accreditation for *N. caninum* is generally not perceived as an added value by farmers. This was confirmed by the relatively low subscription rate for the voluntary *N. caninum* scheme reported by the major herd health scheme providers (CHeCS *Neospora* subgroup, personal communication). The collection of additional information by conducting further questionnaire-based or focus group studies may help to clarify the reasons for such low interest in the accreditation for bovine neosporosis. However, it is possible to speculate that this is likely due to the presence of more pressing health and production issues in modern cattle herds. These include general hypofertility, mastitis and lameness.

According to the sample of veterinarians interviewed, the lack of a vaccine, the farmers' attitude to the problem and the difficulties in interpreting serological test results were the major constraints to the effective control and management of the disease.

In agreement with the consensus in the research field, a vaccine was identified as the most desirable prospective control tool for bovine neosporosis. Although the lower interest displayed towards the use of a prospective antiprotozoal treatment may be attributed to the likely need for withdrawal periods, which may reduce the cost-effectiveness of this option, the specific justifications for the preferential use of a vaccine instead of the administration of an antiprotozoal drug were not investigated. In questionnaire-based surveys, voluntary response bias occurs frequently, since the responders are self-enrolled. This potentially results in the selection of those responders who are more interested and may have a better knowledge of the specific topic covered. It would be difficult to determine whether the majority of veterinarians undertaking the present survey were those with a deeper knowledge of neosporosis than non-responders. It is most likely that all of the responders had direct experience of clinical neosporosis in cattle and were involved in the diagnosis and implementation of control measures. Nevertheless, the questionnaire-based survey presented here provides an interesting insight into the current practice and attitudes towards the diagnosis and control of bovine neosporosis.

5.5 Conclusions

Besides highlighting the need for more reliable serological diagnostics, the questionnaire-based survey evidenced the demand for enhanced guidance in the interpretation of test results, especially those obtained with serological assays.

Continuing professional development (CPD) programmes for large animal veterinarians may be required to update on the characteristics and limitations of the diagnostic options, help the interpretation of the outcome of different tests and standardise the management advice given to farmers. A nationwide industry stakeholder group, similar to the COWS (Control of Worms Sustainability) and the SCOPS (Sustainable Control of Parasites in Sheep) initiatives which promote best practice in the control of ruminants' parasites, may be created for providing veterinarians and farmers with up to date evidence-based information and learning opportunities related to the diagnosis and control of bovine neosporosis.

Finally, vaccination emerged as the preferred control strategy among the veterinarians interviewed suggesting the presence of a potentially wide market for an effective prophylactic product for bovine neosporosis in the United Kingdom.

Chapter 6: General discussion

N. caninum is recognised as a major infectious agent of abortion in dairy and beef cattle responsible for important economic losses and causing animal welfare concerns worldwide (reviewed in Dubey *et al.*, 2017, Reichel *et al.*, 2013).

Over the last three decades, significant research efforts have been invested to study *N. caninum* and bovine neosporosis with the ultimate objective of developing cost-effective strategies to minimise the impact of the disease on cattle production.

As described in Chapter 1, numerous biological features of the parasite as well as host-parasite interaction mechanisms have been investigated and characterised. However, the host-parasite relationship during *N. caninum* infection and the pathogenesis of abortion are still not completely understood (Canton *et al.*, 2013, Dubey *et al.*, 2006a). Abortions not only occur in cows that have been recently exposed to the parasite but also in persistently infected animals following recrudescence of quiescent infections. Although complex interactions between *N. caninum* and the host's immune system are recognised to play a major role, the exact mechanisms underlying the establishment of persistent infections, the long-term survival of the parasite while enclosed in tissue cysts and the reactivation of the bradyzoite stage have not been entirely clarified to date.

Prophylactic and therapeutic options for neosporosis have been investigated (reviewed in Hemphill *et al.*, 2016, Horcajo *et al.*, 2016). Nevertheless, the measures available to prevent *N. caninum* abortions as well as horizontal and vertical transmission have been relatively unchanged for many years (Reichel *et al.*, 2014). The lack of licensed vaccines and antiprotozoal treatments to prevent infection and/or abortion represents a primary constraint to the control of the disease. Even if available, drug treatment of *N. caninum* would carry the risk of residues in milk and meat and the likely need for long-term therapies would render this option uneconomical (Dubey and Schares 2011). Vaccination is therefore regarded as the most desirable control measure. In the past few years, a vaccine based on killed *N. caninum* tachyzoites was licenced for use in cattle in certain countries but subsequently withdrawn from the market due to its limited efficacy in preventing vertical transmission (Weston *et al.*, 2012).

Consequently, current control strategies are restricted to management practices (Reichel *et al.*, 2014).

In this context, the discrimination between infected and uninfected cattle through serology plays a key role. Since clearance of *N. caninum* from infected animals is thought to be highly unlikely, the detection of specific antibodies is not only indicative of exposure to the parasite but also of infection and increased risk of abortion (Weston *et al.*, 2005).

Numerous diagnostic tests for the detection of specific antibodies have been proposed and validated; however, limitations in the serodiagnosis of *N. caninum* have been reported (Dubey *et al.*, 2007, Sinnott *et al.*, 2017).

In *N. caninum*-infected cattle, false-negative results are not only attributable to the characteristics of the serological assays used but can also arise due to the changing dynamics of the host-parasite interaction. Fluctuations of *N. caninum*-specific antibody titres during pregnancy are well-documented (Guy *et al.*, 2001, Jenkins *et al.*, 2002, Waldner *et al.*, 1998). Moreover, in persistently infected cattle, antibody responses elicited by the tachyzoite stage may decline following conversion to the quiescent bradyzoite stage which displays a different antigenic repertoire (Aguado-Martinez *et al.*, 2008, Campero *et al.*, 2017, Guido *et al.*, 2016). Since all commercially available serological tests for the detection of *N. caninum*-specific antibodies employ tachyzoite antigens and predominantly whole tachyzoite extract (Alvarez-Garcia *et al.*, 2013, Campero *et al.*, 2017, von Blumroder *et al.*, 2004) they may fail to identify those persistently infected animals that, despite being infected with *N. caninum*, do not show detectable antibody levels against the tachyzoite stage.

The interesting observation of an infected cow whose antibody responses recognised bradyzoite-specific but not tachyzoite-specific antigens raised concerns about the sensitivity of tests based only on tachyzoite antigens (Benavides *et al.*, 2012). Despite the post-mortem demonstration of the parasite in several tissues, this animal repeatedly tested seronegative with commercial and experimental tests based on tachyzoite antigens; however, seropositivity was detected using an experimental ELISA based on the bradyzoite-specific recombinant antigen NcSAG4.

Antigens specifically expressed by the bradyzoite stage of *N. caninum*, such as NcSAG4 (Fernandez-Garcia *et al.*, 2006), can be successfully employed for the

identification of persistently infected cattle (Aguado-Martinez *et al.*, 2005). Additionally, the detection of bradyzoite-specific antibody responses in parallel with tachyzoite-specific antibody levels enables the discrimination of cattle which have experienced acute primary infection, reinfection, recrudescence of infection or are persistently infected (Aguado-Martinez *et al.*, 2008).

Nevertheless, none of the bradyzoite-specific antigens identified, characterised and used for the serodiagnosis of bovine neosporosis have been made commercially available to date and tools that would enable the detection of persistently infected cattle, which test seronegative with serological tests based on tachyzoite antigens, are currently lacking in the market. In addition, the potential benefits of combining both tachyzoite and bradyzoite-specific antigens to overcome a potential lack of sensitivity of tests based exclusively on tachyzoite antigens have not been fully investigated.

Would a diagnostic test using tachyzoite and bradyzoite antigens enable the reliable identification of *N. caninum* infected cattle?

One of the main aims of this thesis was to further evaluate the use of bradyzoite-expressed antigens for the detection of cattle persistently infected with *N. caninum*.

In Chapter 2, the identification and production of two novel bradyzoite-expressed recombinant antigens was described. Recombinant tNcSRS12A-B and tNcSRS44-A were employed for the detection of *N. caninum*-specific IgGs in cattle that, despite harbouring *N. caninum*, consistently tested antibody-negative with serological assays based on tachyzoite antigens. Preliminary data demonstrated that both antigens may be potentially useful for the identification of persistently infected animals. However, further validation using adequate panels of well-characterised reference sera, which should include samples from naturally infected cattle with persistent infections, are required. At present, such panels are difficult to generate. The characterisation of samples from persistently infected animals, which test antibody negative with assays based on tachyzoite antigens, may be limited because of the lack of tests based on antigens expressed by the bradyzoite stage. For the same reason, the selection of seronegative reference samples may require very strict criteria to rule out that they are obtained from animals which test negative with current tachyzoite-based assays despite being persistently infected with *N. caninum*. These criteria include the

selection of seronegative animals from maternal family lines in which no evidence of exposure to the parasite is observed (i.e. consistent seronegative results in several generations). Such animals should be selected from herds with no history of abortions and serological positive results for *N. caninum*.

Furthermore, standardised models of persistent infection in ruminants are currently unavailable (Benavides *et al.*, 2014). Although cattle that become infected with *N. caninum* are considered persistently infected for life, the development of experimental models, in which doses, route of administration, strain of *N. caninum* and long-term time points in which serological testing is carried out, may help to identify and characterise patterns in the antibody levels against different stage-specific antigens. Such models should include the periodic serological examination of experimentally infected pregnant dams during prolonged periods and subsequent pregnancies. This may enable a fuller picture to be obtained on the effects that the changes in the host-parasite interaction may have on the detection of antibody levels against antigens expressed by different lifecycle stages of *N. caninum*.

Overall, the inadequacy of serological tools based on bradyzoite-specific antigens and the lack of experimental models of persistent infection may be responsible for the limited knowledge regarding the humoral immune responses elicited by the bradyzoite stage of *N. caninum* during persistent infections.

Acute infection with *N. caninum* results in the production of specific immunoglobulins against the tachyzoite stage of the parasite which is readily exposed to the host's immune system since it rapidly proliferates and spreads throughout the body of infected animals (Williams *et al.*, 2000). During persistent infections, recurrent rupture of tissue cysts with exposure of bradyzoites may be necessary to induce detectable antibody responses against antigens expressed by the bradyzoite stage, as suggested for the closely related apicomplexan *T. gondii* (Gross *et al.*, 2004). There is limited information on the *in vivo* biology of *N. caninum* tissue cysts. Studies carried out in rodents infected with *T. gondii* showed that tissue cyst rupture occurs consistently over the lifetime of infected animals. In experimentally infected rats, this was not necessarily associated with reactivation from the bradyzoite to the tachyzoite stage (Dubey *et al.*, 2016). In this way, bradyzoite antigens would be exposed to the effector cells of the host's immune system, with subsequent production of specific antibodies,

without an increase of antibody titres against antigens displayed by the tachyzoite stage.

Similarly to previous studies (Aguado-Martinez *et al.*, 2008), in Chapter 2 and 3, antibody responses against bradyzoite antigens were successfully detected and considered as markers of persistent infection in cattle. Nonetheless, the number and localisation of tissue cysts, which are expected to be highly variable depending on the individual animal and virulence of the *N. caninum* isolate involved, as well as the frequency with which these cysts would rupture during persistent infections are currently unknown. Further studies are required to assess the level of exposure of *N. caninum* bradyzoite antigens to the bovine immune system. For this purpose, the development of ruminant models of persistent neosporosis may be explored.

Antibody ELISAs based on tNcSRS12A-B and tNcSRS44-A would have enabled the further assessment of the diagnostic potential of these antigens; however, technical issues related to the physico-chemical conditions in which they were produced hindered the development of such tests. Subsequent attempts to obtain useable ELISAs, including the use of different expression and purification systems, did not prove successful. Nevertheless, WBs using tNcSRS12A-B and tNcSRS44-A may be beneficial to further characterise the serological status of cattle which test antibody negative with tests based on tachyzoite antigens. Antibodies detected with the tNcSRS12A-B and tNcSRS44-A WBs may suggest persistent infection whilst negative antibody results using these tests would enable the classification of *N. caninum* seronegative cattle more confidently. Notably, the application of these serological tools may be helpful when thorough serological analysis is required; for example, within the selection of breeding stock in high genetic merit herds in which the increased costs of the additional diagnostic methods would be justified. In vaccine studies, the use of improved tests using bradyzoite-specific antigens would increase the confidence in selecting truly naïve experimental animals and reduce the risk of enrolling persistently infected cattle which may test seronegative with current tachyzoite antigens-based tests. Previous exposure to the parasite would impact negatively vaccination experiments in which the evaluation of the immune responses in naïve cattle following a primary challenge is required.

In Chapter 3, one commercial and five experimental antibody ELISAs based on stage-specific antigens (Aguado-Martinez *et al.*, 2008, Risco-Castillo *et al.*, 2007, Risco-Castillo *et al.*, 2011) were evaluated. All of the ELISA tests were adapted and reassessed to measure seroprevalence to *N. caninum* in a sample of British dairy cattle. The fitness for purpose should be the guiding principle for the application of a serological test to a specific situation (Dubey *et al.*, 2017). The assessment and comparison of the diagnostic performances was limited by the absence of a true gold standard technique and a well-characterised panel of reference plasma samples as the *N. caninum* antibodies were measured in plasma instead of serum. These limitations were overcome by using a non-gold standard approach for the selection of the optimal cut-off to determine seropositive and seronegative animals.

Assays using whole native and recombinant tachyzoite antigens showed better performances compared to tests based on recombinant bradyzoite-specific antigens. Apart from suggesting the need for further optimisation of the tests based on bradyzoite-specific antigens, this may reflect the variability in the intensity of the antibody responses against the antigens expressed by the bradyzoite stage which may be due to the variable level of exposure of these antigens to the host immune system. As previously discussed, this aspect of the host-parasite relationship of *N. caninum* is not well understood and supplementary studies investigating the antibody responses to bradyzoite-specific antigens are required. For example, the longitudinal serological assessment of persistently infected animals using assays based on several recombinant bradyzoite-specific antigens may help to clarify the frequency and intensity of the development of antibody responses to these antigens. The recombinant antigens tNcSRS12A-B and tNcSRS44-A described in this thesis may be included in the longitudinal serological analyses to complement the information obtained with other assays using bradyzoite-specific antigens of *N. caninum* which have been previously reported in literature.

ELISAs using antigens expressed by different lifecycle stages of *N. caninum* showed only slight agreement. At the same time, moderate agreement was observed amongst assays based on tachyzoite immunodominant antigens, either native or recombinant, and amongst bradyzoite-specific antigen-based tests. This suggested that the two types of antigenic preparation are recognised by different antibody responses against *N.*

caninum. These two aspects do not necessarily overlap supporting the hypothesis that tests based exclusively on tachyzoite antigens may result in the misclassification of a proportion of infected animals as uninfected. The estimation of this proportion strictly depends on the test or combination of tests used.

A single commercial test based on *N. caninum* whole tachyzoite extract misclassified as seronegative 46.8% (95%CI: 39.5-54.2) of the samples considered positive based on the outcome of all six ELISAs assessed. Similar proportions were obtained considering the outcome of individual ELISAs based on recombinant tachyzoite antigens (rNcSRS2 and rNcGRA7). When the results of all assays based on tachyzoite antigens were considered collectively, the proportion dropped to 18.5% (13.4-24.9). This figure may be considered a preliminary estimated of the proportion of cattle that despite being infected with *N. caninum* and showing detectable antibody responses to bradyzoite-specific antigens would test seronegative with tachyzoite antigens-based assays.

However, careful interpretation is required since tests based on tachyzoite antigens, either native or recombinant, evaluated individually, also produced numerous false negative results. This was likely due to the moderate agreement observed between tests based on antigens expressed by the same stage of *N. caninum* which reflected the different antigenic preparations and technical characteristics of each ELISA.

Consequently, the frequency and hence the epidemiological significance of persistently infected animals which would not be currently identified using assays based only on tachyzoite antigens is difficult to assess.

When followed by the appropriate management of seropositive animals, the serodiagnosis using current commercial or in-house ELISAs, mostly based on native *N. caninum* tachyzoite antigens, can significantly reduce the impact of bovine neosporosis in those herds in which effective biosecurity and hygienic measures are implemented (McAllister 2016). However, the data produced in this thesis confirmed the concerns about the shortcomings of currently available serological diagnostic tools. The occurrence of false negative results should be considered when screening cattle for *N. caninum* antibodies and, where possible, a combination of tests executed in parallel or a single serological assay combining antigens expressed by different lifecycle stages of *N. caninum* should be advised.

The combination of 4 tests (3 tachyzoite antigen-based and 1 bradyzoite antigen-based ELISAs) provided satisfactory levels of sensitivity and specificity (>90%). In the absence of assays characterised by improved performances, the application of these tests in parallel should be considered as an effective strategy to increase the diagnostic sensitivity of the serological diagnosis of *N. caninum* in cattle.

On-farm control programmes for bovine neosporosis would greatly benefit from the increased diagnostic sensitivity. Animals which may not test seropositive with current tachyzoite antigen-based assays, but are infected, may be identified and appropriately managed (i.e. culling or selective breeding) to reduce the prevalence of the disease at the herd level.

Epidemiological information with practical implications

Due to the different performances and characteristics of the tests and combination of tests evaluated, the seroprevalence of *N. caninum* in British dairy cattle varied considerably depending on the serological tool considered. The between-herd seroprevalence observed was high (>65%), when assessed using a single commercial test, or very high (>90%) considering the outcome of different combinations of tests, confirming that *N. caninum* is widespread in British dairy herds.

Interestingly, the cross-sectional seroprevalence study enabled the evaluation of serologically testing cattle for *N. caninum* in different productive/reproductive stages. No statistically significant differences between the seroprevalence observed in animals during the dry period (i.e. during late pregnancy) and cows in mid- and early lactation were observed suggesting the lack of evidence for restricting testing during late pregnancy.

In the UK, current health schemes recommend carrying out serological screening for *N. caninum* in cows between 12 and 4 weeks pre-calving (CHeCS 2016). This sampling window would be justified by the rise of *N. caninum*-specific antibody titres after the fifth month of gestation (Andrianarivo *et al.*, 2005, Cardoso *et al.*, 2009, Dannatt 1997, Guy *et al.*, 2001) which would increase the probability of detecting infected animals. Based on this assumption a significantly higher seroprevalence in dry cows (late gestation) compared to cows in early lactation (non-pregnant) or mid-gestation (early pregnancy) was hypothesised. However, this was not observed

irrespective of the single test or combination of tests used for estimating the seroprevalence. Since most of the dry cows tested were sampled during the last two weeks prior to the expected calving date, the physiological drop in the concentration of the total IgGs in peripheral blood observed in bovines close to calving (Detilleux *et al.*, 1995, Franklin *et al.*, 2005) may have resulted in a lower seroprevalence observed in this group.

In summary, the validity of restricting the serological testing of cows during late pregnancy was not confirmed suggesting that different sampling regimes, which include non-pregnant cows or animals in early gestation, may not affect the reliability in identifying infected animals when serological screening campaigns are carried out within control programmes. However, further cross-sectional studies including a larger number of cows sampled at earlier stages in the dry period (i.e. during late pregnancy but not close to parturition) may be useful to confirm this finding. Supplementary longitudinal studies to assess whether cows in late pregnancy have a significantly higher probability of testing seropositive compared to cows at different reproductive stages would also be useful.

Application of a MLFT tool for understanding the epidemiology of neosporosis

In Chapter 4, a MLFT tool was optimised and successfully applied to the study of the genetic diversity of *N. caninum*. In general, the 12 microsatellite markers proposed were species-specific and showed good discriminatory power and typeability. The microsatellite markers proposed may contribute to the creation of an internationally recognised MLFT tool which would capitalise on the different panels of micro- and minisatellite *loci* previously described in literature (Al-Qassab *et al.*, 2010a, Regidor-Cerrillo *et al.*, 2006); this may require an international workshop to select and define optimal markers.

In the research field, the possible applications of this tool encompass studies of the population genetics of *N. caninum* and the discrimination of vaccine and challenge isolates within the development of live vaccines (Basso *et al.*, 2010, Goodswen *et al.*, 2013, Regidor-Cerrillo *et al.*, 2013).

In clinical practice, the MLFT can be used for the discrimination of different isolates involved in abortion outbreaks. In this context, the study of the genetic diversity can be very useful for providing additional information on the source of infection and the predominant route of transmission at the herd level (Basso *et al.*, 2010). Additionally, the detection of identical *N. caninum* genotypes in cattle and wildlife living in the same environment, in which definitive canine hosts are present, may enable the identification of potential reservoirs of the parasite.

Support for veterinarians in the interpretation of serological diagnostics

Finally, the questionnaire-based survey for large animal veterinarians described in Chapter 5 provided an interesting insight into current veterinary practice related to the diagnosis and management of bovine neosporosis in the United Kingdom.

Concerns about the difficult interpretation of the results obtained with currently available serological tests and the occurrence of false negative results appeared from the survey. Besides highlighting the need for improved diagnostics, these findings supported the demand for continuing professional training opportunities for both veterinarians, regarding the interpretation of test results and evidence-based measures for managing *N. caninum* infections effectively, and farmers, in order to clarify the infection mechanisms and transmission routes of the parasite.

An effective vaccine was indicated as the control tool which would have the highest beneficial impact on the management of bovine neosporosis in future. Besides indicating the existence of a potential wide market for a commercial product, the consensus on the requirement for a prophylactic option strongly supported the view that vaccine development should be a primary focus in *N. caninum* research (Marugan-Hernandez 2017, Nishikawa 2017). New avenues for the identification of potential vaccine candidates, including *in silico* methods which capitalise on the great wealth of genomic, transcriptomic and proteomic information available, should therefore be explored further (Goodswen *et al.*, 2014a, Goodswen *et al.*, 2014b, Goodswen *et al.*, 2017).

6.1 Concluding remarks

Management-based control programmes are currently the only option to reduce the impact of *N. caninum* in cattle herds worldwide (Reichel *et al.*, 2014). While there is consensus that vaccination would enable the effective and economically sustainable control of bovine neosporosis, an effective vaccine may not be available for several years to come (Reichel *et al.*, 2013).

The optimisation and refinement of current control programmes would have an immediate positive impact on the control of the disease at the herd, regional and national levels.

Integrated high health schemes are the way forward to control bovine neosporosis. These should combine recommendations on evidence-based biosecurity and hygienic measures to prevent the introduction and transmission of *N. caninum* as well as guidance on the characteristics of the diagnostic tests to be used and their application in different situations (i.e. confirmation of neosporosis in aborting dams, diagnosis of the disease in non-aborting animals and assessment of the within-herd prevalence).

Serology-based diagnostics play a key role in informing on the infection status of individual animals. The data presented in this thesis highlighted the shortcomings of current tests based on tachyzoite antigens, the need for the development of improved serological tests and suggested that using a wider range of *N. caninum* antigens, expressed by different lifecycle stage of the parasite, may be a valid strategy to increase diagnostic sensitivity.

In the absence of a single serological method able to detect also those animals which would not be currently identified with assays based on tachyzoite antigens, the combination of multiple tests based on antigens expressed by both the tachyzoite and bradyzoite stages of *N. caninum* can be used to increase the sensitivity. Serological tools combining three ELISAs based on tachyzoite antigens and one assay based on a recombinant bradyzoite-specific antigen showed good sensitivity and specificity suggesting that a combination of tests, carried out in parallel, should be considered to improve the serological diagnosis of bovine neosporosis.

In uninfected herds, serological tests characterised by high diagnostic sensitivity are essential for testing prospective purchased breeding cattle to prevent the introduction

of *N. caninum*. Likewise, in *N. caninum* problem herds, the management of neosporosis would benefit enormously from improved diagnostics as these will not only enable the reliable identification of infected animals but also allow the accurate estimation of the within-herd seroprevalence. Indeed, knowing the seroprevalence of *N. caninum* in a herd is fundamental to carry out the cost-benefit analysis which is a prerequisite for preferring a specific control option rather than a different one (e.g. culling instead of selective breeding in herds with low prevalence).

Furthermore, the investigation of *N. caninum* abortion outbreaks using molecular typing tools to study the genetic diversity of the parasite may help in shaping effective control strategies by providing useful epidemiological information related to the source of infection and the predominant transmission route.

Collectively, the reliable identification of *N. caninum*-infected cattle and the availability of supplementary in-depth epidemiological information would enable large animal veterinarians to provide farmers with the optimal advice on the best course of action to tackle neosporosis.

Effective management of the disease obtained using improved diagnostics will be of great benefit to the cattle sector as it will result in enhanced livestock health and welfare and increased productive efficiency in both dairy and beef farms worldwide.

Alongside vaccine research, which should be a priority, the development of innovative diagnostics characterised by high accuracy, reduced cost and short execution time should be supported (Ghalmi *et al.*, 2014). For example, rapid pen-side immunochromatographic tests, employing *N. caninum* stage-specific antigens, may be investigated as an option to reduce execution times and costs of the serological diagnosis.

In future, *in silico* tools currently applied to the selection of suitable vaccine candidates may be employed for the identification of target proteins and peptides that may be used for designing novel epitope-based serological diagnostics (Bui *et al.*, 2007, Goodswen *et al.*, 2014a, Goodswen *et al.*, 2014b).

Finally, the questionnaire-based survey highlighted that specialist cattle veterinary practitioners and farmers would benefit from knowledge exchange opportunities to discuss the most up to date information available on the diagnosis and control of bovine neosporosis.

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Appendix I

General laboratory practice and solutions

PCR and cloning reagents

Tris-acetate-EDTA (TAE) buffer

The following reagents were combined to prepare a concentrated 50 × TAE solution:

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

dH₂O to 1 litre.

DNA loading buffer

A 6 × concentrated gel loading buffer was made by combining the following:

15 % v/v Fycoll® (type 400) in dH₂O

0.25 % bromophenol blue

0.25 % xylene cyanol FF

The loading buffer was stored at 4°C.

1.8% agarose gel

3.6 g agarose were combined with 1 × TAE buffer to a final volume of 200 ml and heated on full power in a microwave until complete dissolution of the agarose. The mix was cooled to approximately 45°C before adding 12 µl 10,000 × Gel Red™ (Biotum, Hayward, CA, USA). The gel was then poured into an electrophoresis tank with pre-positioned combs to give the number and size of wells required. After solidification, the combs were removed and the gel was submerged with 1 × TAE. DNA samples combined with DNA loading buffer and a 1 kb DNA ladder (Promega, Madison, WI, USA) were then loaded into the relevant wells. The tank was attached to a power supply. 100 V were applied for approximately 45 mins and the gel visualised using UV.

LB-Broth (Luria-Bertani Medium)

10 g Bacto®-tryptone

5 g Bacto®-yeast extract

10 g NaCl

The volume was made up to 1 litre with distilled water and the pH adjusted to 7.5 with NaOH. This was then autoclaved to sterilise.

For LB agar plates, 15 g agarose were added prior to autoclaving.

Solutions for recombinant protein purification

Phosphate-buffered saline (PBS)

0.8 g NaCl

0.2 g KCl (potassium chloride)

1.8 g Na₂HPO₄·2H₂O (sodium phosphate dibasic dihydrate)

0.3 g KH₂PO₄ (monopotassium phosphate)

sterile dH₂O to 1 litre.

Autoclave at 121°C for 15 min.

200 mM sodium phosphate buffer monobasic (NaH₂PO₄)

4.8 g NaH₂PO₄

sterile dH₂O to 200 ml.

200 mM sodium phosphate buffer dibasic (Na₂HPO₄)

14.25 g Na₂HPO₄

sterile dH₂O to 100 ml.

2 M imidazole

13.62 g imidazole

sterile dH₂O to 500 ml.

Binding buffer 1

5.7 g 200mM NaH₂PO₄

24.3 g 200mM Na₂HPO₄

8.7 g NaCl

sterile dH₂O to 300 ml.

Binding buffer 2 (8M urea)

5.7 g 200mM NaH₂PO₄

24.3 g 200mM Na₂HPO₄

8.7 g NaCl

144.14 g urea

sterile dH₂O to 300 ml

Sterile dH₂O was warmed up to ease urea dissolution.

Denaturing equilibration buffer

50 ml 10 × PBS

2.5 ml 2M imidazole

242.5 g urea

sterile dH₂O to 500 ml.

Denaturing wash buffer

50 ml 10 × PBS

6.25 ml 2M imidazole

242.5 g urea

sterile dH₂O to 500 ml.

Denaturing elution buffer

50 ml 10 × PBS

62.5 ml 2M imidazole

242.5 g urea

sterile dH₂O to 500 ml.

Dialysis buffer 1 (1M urea)

60.06 g urea

1 × PBS to 1 litre.

Dialysis buffer 2 (2M urea)

120.12 g urea

1 × PBS to 1 litre.

Dialysis buffer 3 (4M urea)

240.24 g urea

1 × PBS to 1 litre.

Solutions for Western Blot**Transfer buffer**

50 ml NuPAGE® transfer buffer 20 ×

1 ml NuPAGE® antioxidant

200 ml methanol

dH₂O to 1 litre.

Blocking buffer (4% dried skimmed milk)

58.44 g NaCl

10 ml Tween® 80

20 g dried skimmed milk (Marvel)

1 × PBS to 500 ml

Filter, aliquot in 15 ml aliquots and store at -20°C.

Wash buffer

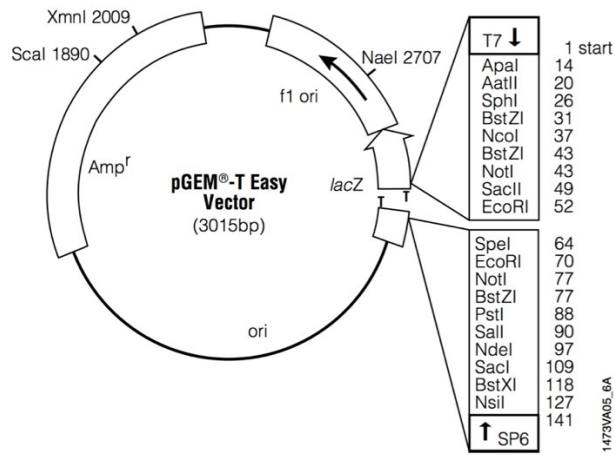
58.44 g NaCl

10 ml Tween® 80

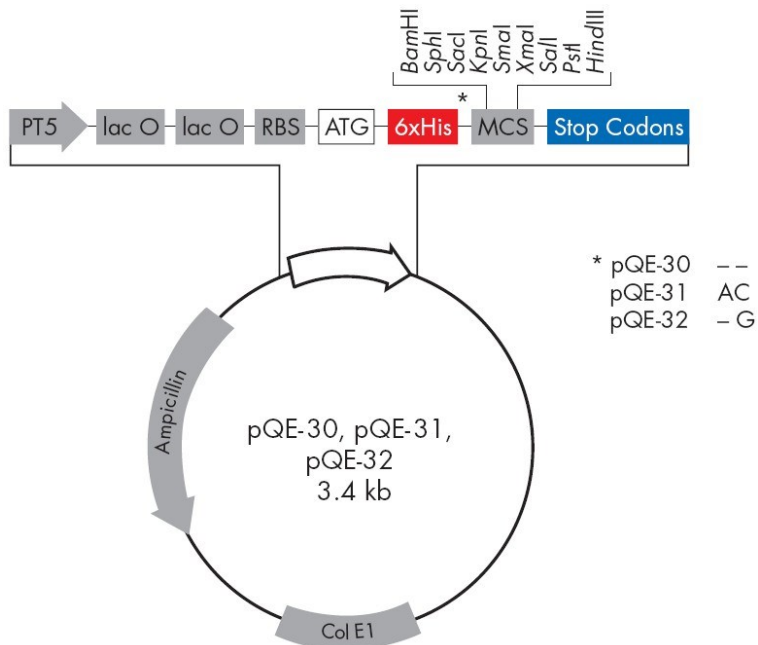
dH₂O to 2 litres.

Appendix II

pGEM®-T Easy vector map and sequence reference points



pQE-30, pQE-31 and pQE-32 vectors map



Appendix III

Questionnaire for cattle veterinary practitioners (final version)

Diagnosis and control of *Neospora caninum* infection in cattle: a questionnaire for cattle veterinary practitioners

Approaching the diagnosis of bovine neosporosis and deciding the best course of action to control the disease and prevent future infections can be problematic sometimes. The present survey will take approximately 20 minutes to complete and will allow us to analyse how veterinary practitioners perceive bovine neosporosis and what are their attitudes towards the diagnosis and control of the disease.

Section 1 – General assessment and perceptions

1) Which **infectious causes of bovine abortion** are the most frequently diagnosed in your area? (please, rank in descending order of frequency the top 3 (from 1 = 1st most frequently diagnosed to 3 = 3rd most frequently diagnosed)

1.
2.
3.

Which one would you rate as the most challenging in terms of diagnosis?
.....

Which one would you rate as the most challenging in terms of control?
.....

2) According to your personal experience, how would you rate the economic impact of **bovine neosporosis** in cattle herds?

- | | |
|-----------|--------------------------|
| Very low | <input type="checkbox"/> |
| Low | <input type="checkbox"/> |
| High | <input type="checkbox"/> |
| Very high | <input type="checkbox"/> |

3) The following statements will cover **your perceptions of *N. caninum* and bovine neosporosis**.

Please, state if you **strongly agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
I feel confident about my understanding of bovine neosporosis and <i>N. caninum</i> life cycle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<i>N. caninum</i> infection in cattle can be very difficult to control	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of bovine neosporosis is important for the profitability of dairy farms	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of bovine neosporosis is important for the profitability of beef suckler herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Farmers perceive <i>N. caninum</i> as a major threat to their farming businesses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Farmers understand <i>N. caninum</i> life cycle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of <i>N. caninum</i> is important to the cattle farming industry as a whole	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Sometimes I have doubts about how to interpret <i>N. caninum</i> diagnostic tests	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Positive results produced with current diagnostics are trustworthy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Negative results produced with current diagnostics are trustworthy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Section 2 – Diagnosis of bovine neosporosis

4) When do you start to be concerned about *N. caninum* in a herd (i.e. consider doing further testing specific for the parasite)? (select all that apply)

- Abortion storm on farm
- Abortion storms in neighbouring farms
- One or more confirmed *N. caninum* abortion case/s
- Ongoing endemic *N. caninum* abortions
- Other, please specify

5) a. How do you deal with *N. caninum* **abortion suspects**?

.....
 b. What test options give you most confidence to make a diagnosis of neosporosis?

6) a. How do you assess the *N. caninum* **infection status** (present or absent) in live animals in a herd?

.....
 b. Which animals do you sample and what samples do you collect?

c. What diagnostic tests do you use?

7) The following statements will cover the **assessment of *N. caninum* infection at the herd level (present or absent)**.

Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Antibody ELISA on bulk milk is the most cost-effective way to assess whether a herd is infected or not	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Negative bulk milk testing results mean that the herd is <i>N. caninum</i> free	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted foetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
ELISA testing sera from animals in the sampling window (12 to 4 weeks pre-calving) is preferable to bulk milk testing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted foetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

None of the available diagnostic tests can be considered as a gold standard for bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
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8) The following statements cover the **assessment of *N. caninum* infection at the herd level (present or absent)**.

Please, state **how frequently** you undertake the following procedures.

Always (A), Frequently (F), Rarely (R), Never (N)

	A	F	R	N
I bleed animals within the sampling window (12 to 4 weeks pre-calving) to test for specific <i>N. caninum</i> antibodies	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In herds with <i>N. caninum</i> problems, I recommend carrying out serum antibody ELISA test on all dry cows	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I consider cows to be <i>Neospora</i> negative if they test negative twice for <i>N. caninum</i> antibodies, when tested in the sampling window over two pregnancies	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I recommend submission of aborted fetuses and placentas to a veterinary laboratory for foetal serology and histopathological examinations	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I recommend serology testing for <i>N. caninum</i> in aborting dams	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
When a <i>N. caninum</i> abortion is confirmed, I sample all the animals in the same cohort as the dam that aborted	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

9) The next statements will cover the **diagnosis of *N. caninum* abortion cases**.

Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
PCR positive results require foetal and/or dam serology to confirm <i>N. caninum</i> as the cause of the abortion	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Blood testing dams that have recently aborted with serum antibody ELISA is essential to confirm <i>N. caninum</i> as the cause of abortion when evidence is found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

10) Do current *Neospora* serology based diagnostics (ELISAs) give reliable information?

Yes

No

Unsure

a. Why or why not?

.....

b. Is the information obtained from serology based diagnostic tests useful? Why?

.....

Section 3 – Control strategies

11) Has your practice got a common/agreed protocol for the diagnosis and advices on the control of bovine neosporosis?

Yes

No

12) If yes, is it based on what is indicated by a herd health scheme provider?

Yes

No

If yes, which is the herd health scheme provider?

13) Do you consider testing farm dogs prior starting control programmes for bovine neosporosis?

Yes

No

If yes, would you:

Do serology for *N. caninum* specific antibody titres

Test faeces for *N. caninum* oocysts

14) The following statements will examine **biosecurity measures** for bovine neosporosis. Please, state if you **strongly agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Good biosecurity practice is essential for the control of bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Biosecurity measures should be aimed at minimizing horizontal transmission (ingestion of <i>N. caninum</i> oocysts shed by dogs)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
The prompt removal and proper disposal of afterbirths is seen as a priority in most dairy herds during calving	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
The prompt removal and proper disposal of afterbirths is seen as a priority in most beef sucklers herds during calving	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Vermin control still represents an issue in most farms	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Despite a good biosecurity plan, <i>N. caninum</i> can be easily introduced in uninfected herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Herd biosecurity plans should include measures that are specific for the control of bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Regardless of their serological status, dogs should be kept out of calving areas, feed facilities and pastures	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Increased public engagement is needed in order to make dog owners aware of the potential risk their dogs could represent to cattle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Buying in replacement animals is one of the most likely ways to introduce <i>N. caninum</i> to an uninfected herd	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

15) Do you recommend testing **prospective purchased heifers and cows** for *N. caninum* prior introducing them into the herd?

Always

Frequently

Rarely

Never

16) The following statements will cover **control strategies** for bovine neosporosis.

Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Bovine neosporosis should be included in herd health schemes	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
As for other infection diseases, farmers perceive accreditation for <i>N. caninum</i> as an added value to their businesses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

17) The following statements will cover **control strategies** for bovine neosporosis.

Please, state **how frequently** you undertake the following procedures.

Always (A), Frequently (F), Rarely (R), Never (N)

	A	F	R	N
In farms with seroprevalence >10%, I recommend selective breeding of heifers from dams that test <i>N. caninum</i> antibody negative	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In farms with seroprevalence <5%, I recommend culling of seropositive animals	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I would advise the use of Embryo Transfer to seronegative recipients from high genetic merit cow donors because they are <i>N. caninum</i> seropositive	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I consider the "Live with the disease - Do nothing" option as a possible approach in those herds in which the impact (abortion cases) is low	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
If available, I would recommend a vaccine to prevent <i>N. caninum</i> infections and/or abortions	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
If licensed, I would recommend antimicrobials to prevent <i>N. caninum</i> abortions in problem herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

18) Which are the key points you emphasize when informing and advising farmers about the control of bovine neosporosis?

.....

19) In your opinion, what are the major limitations in the control of bovine neosporosis?

.....

Vet details

20) What is your primary activity?

Veterinary surgeon in private practice

Veterinary investigation officer/consultant (e.g. employee of APHA, SRUC/SAC)

Diagnostics/Research

21) How old are you?

24-35

36-50

51-65

Over 65

22) How many years have you been working as a farm animal veterinary practitioner?

5 years or less

5-10

10-20

20- 30

40 or more

23) Please, state your field of activity within the cattle sector

Dairy

Beef

Mixed

Mixed mainly dairy

Mixed mainly beef

24) Where is your practice located?

Postcode:

Questionnaire for cattle veterinary practitioners (trial version)

Questions removed or reworded are marked in red.

Diagnosis and control of *Neospora caninum* infection in cattle: a questionnaire for cattle veterinary practitioners

Section 1 – General assessment and perceptions

1) Which **infectious causes of bovine abortion** are the most frequently diagnosed in your area? (please, rank in descending order of frequency the top 3 (from 1 = 1st most frequently diagnosed to 3 = 3rd most frequently diagnosed)

1.
2.
3.

Which one would you rate as the most challenging in terms of diagnosis?
.....

Which one would you rate as the most challenging in terms of control?
.....

2) According to your personal experience, how would you rate the economic impact of **bovine neosporosis** in cattle herds?

- Very low
 Low
 High
 Very high

3) The following statements will cover **your perceptions of *N. caninum* and bovine neosporosis**.

Please, state if you **strongly agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
I feel confident about my understanding of bovine neosporosis and <i>N. caninum</i> life cycle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
<i>N. caninum</i> infection in cattle can be very difficult to control	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of bovine neosporosis is important for the profitability of dairy farms	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of bovine neosporosis is important for the profitability of beef sucklers herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Farmers perceive <i>N. caninum</i> as a major threat to their farming businesses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Farmers understand <i>N. caninum</i> life cycle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Control of <i>N. caninum</i> is important to the cattle farming industry as a whole	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Sometimes I have doubts about how to interpret <i>N. caninum</i> diagnostic tests	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Positive results produced with current diagnostics are trustworthy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Negative results produced with current diagnostics are trustworthy	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Section 2 – Diagnosis of bovine neosporosis

4) When do you start to be concerned about *N. caninum* in a herd (i.e. consider doing further testing specific for the parasite)? (select all that apply)

- Abortion storm on farm
- Abortion storms in neighbouring farms
- Ongoing endemic *N. caninum* abortions
- Other, please specify

*** question altered in the final version**

5) a. How do you deal with *N. caninum* **abortion suspects**?

.....

b. What test options give you most confidence to make a diagnosis of neosporosis?

.....

6) a. How do you assess the *N. caninum* **infection status** in live animals in a herd?

*** question altered in the final version**

b. Which animals do you sample and what samples do you collect?

.....

c. What diagnostic tests do you use?

.....

7) **[Question removed]**

a. How do you assess the **prevalence** in infected herds?

.....

b. Which animals do you sample and what samples do you collect?

.....

c. What diagnostic tests do you use?

.....

8) The following statements will cover the **assessment of *N. caninum* infection at the herd level (present or absent)**.

Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Antibody ELISA on bulk milk is the most cost-effective way to assess whether a herd is infected or not	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Negative bulk milk testing results mean that the herd is <i>N. caninum</i> free	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
ELISA testing sera from animals in the sampling window (12 to 4 weeks pre-calving) is preferable to bulk milk testing	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

None of the available diagnostic tests can be considered as a gold standard for bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
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9) The following statements cover the **assessment of *N. caninum* infection at the herd level (present or absent)**.

Please, state **how frequently** you undertake the following procedures.
Always (A), Frequently (F), Rarely (R), Never (N)

	A	F	R	N
I bleed animals within the sampling window (12 to 4 weeks pre-calving) to test for specific <i>N. caninum</i> antibodies	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In herds with <i>N. caninum</i> problems, I recommend carrying out serum antibody ELISA test on all dry cows	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I consider cows to be <i>Neospora</i> negative if they test negative twice for <i>N. caninum</i> antibodies, when tested in the sampling window over two pregnancies	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I recommend submission of aborted fetuses and placentas to a veterinary laboratory for foetal serology and histopathological examinations	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I recommend serology testing for <i>N. caninum</i> in aborting dams	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
When a <i>N. caninum</i> abortion is confirmed, I sample all the animals in the same cohort as the dam that aborted	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

10) The next statements will cover the **diagnosis of *N. caninum* abortion cases**.

Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
PCR positive results require foetal and/or dam serology to confirm <i>N. caninum</i> as the cause of the abortion	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Blood testing dams that have recently aborted with serum antibody ELISA is essential to confirm <i>N. caninum</i> as the cause of abortion when evidence is found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In some cases, dams that aborted test seronegative despite <i>N. caninum</i> evidence being found in aborted fetuses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

11) Do current *Neospora* serology based diagnostics (ELISAs) give reliable information?

- Yes
- No
- Unsure

- c. Why or why not?
.....
- d. Is the information obtained from serology based diagnostic tests useful? Why?
.....

Section 3 – Control strategies

12) Has your practice got a common/agreed protocol for the diagnosis and advices on the control of bovine neosporosis?

- Yes
- No
- 13) If yes, is it based on what is indicated by a herd health scheme provider?
- Yes
- No
- If yes, which is the herd health scheme provider?
- 14) Do you consider testing farm dogs prior starting control programmes for bovine neosporosis?
- Yes
- No
- If yes, would you:
- Do serology for *N. caninum* specific antibody titres
- Test faeces for *N. caninum* oocysts

- 15) The following statements will examine **biosecurity measures** for bovine neosporosis. Please, state if you **strongly agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Good biosecurity practice is essential for the control of bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Biosecurity measures should be aimed at minimizing horizontal transmission (ingestion of <i>N. caninum</i> oocysts shed by dogs)	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
The prompt removal and proper disposal of afterbirths is seen as a priority in most dairy herds during calving	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
The prompt removal and proper disposal of afterbirths is seen as a priority in most beef sucklers herds during calving	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Vermin control still represents an issue in most farms	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Despite a good biosecurity plan, <i>N. caninum</i> can be easily introduced in uninfected herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Herd biosecurity plans should include measures that are specific for the control of bovine neosporosis	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Regardless of their serological status, dogs should be kept out of calving areas, feed facilities and pastures	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Increased public engagement is needed in order to make dog owners aware of the potential risk their dogs could represent to cattle	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Buying in replacement animals is one of the most likely ways to introduce <i>N. caninum</i> to an uninfected herd	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

- 16) Do you recommend testing **prospective purchased heifers and cows** for *N. caninum* prior introducing them into the herd?

- Always
- Frequently
- Rarely
- Never

- 17) The following statements will cover **control strategies** for bovine neosporosis. Please, state if you strongly **agree (SA)**, **agree (A)**, are **unsure (U)**, **disagree (D)** or **strongly disagree (SD)** with the following statements.

	SA	A	U	D	SD
Bovine neosporosis should be included in herd health schemes	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
As for other infection diseases, farmers perceive accreditation for <i>N. caninum</i> as an added value to their businesses	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

18) The following statements will cover **control strategies** for bovine neosporosis.

Please, state **how frequently** you undertake the following procedures.

Always (A), Frequently (F), Rarely (R), Never (N)

	A	F	R	N
In farms with HIGH seroprevalence, I recommend selective breeding of heifers from dams that test <i>N. caninum</i> antibody negative *reworded in the final version	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
In farms with LOW seroprevalence, I recommend culling of seropositive animals *reworded the in final version	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I would advise the use of Embryo Transfer to seronegative recipients from high genetic merit cow donors because they are <i>N. caninum</i> seropositive	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
I consider the "Live with the disease - Do nothing" option as a possible approach in those herds in which the impact (abortion cases) is low	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
If available, I would recommend a vaccine to prevent <i>N. caninum</i> infections and/or abortions	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
If licensed, I would recommend antimicrobials to prevent <i>N. caninum</i> abortions in problem herds	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

19) Which are the key points you emphasize when informing and advising farmers about the control of bovine neosporosis?

.....

20) **When do you advise selective breeding instead of culling? [Question removed]**

.....

21) In your opinion, what are the major limitations in the control of bovine neosporosis?

.....

Vet details

22) What is your primary activity?

Veterinary surgeon in private practice

Veterinary investigation officer/consultant (e.g. employee of APHA, SRUC/SAC)

Diagnostics/Research

23) How old are you?

24-35

36-50

51-65

Over 65

24) How many years have you been working as a farm animal veterinary practitioner?

- 5 years or less
- 5-10
- 10-20
- 20- 30
- 40 or more

25) Please, state your field of activity within the cattle sector

- Dairy
- Beef
- Mixed
- Mixed mainly dairy
- Mixed mainly beef

26) Where is your practice located?

Postcode: