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

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CONTENTS

1. Abstract

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 report 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.

2. Introduction

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.

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 recrudescent 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.

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 recrudescent 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.

Within the present project, 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.

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 foetuses 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.,* 2007b).

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 foetuses (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.,* 2002a, Regidor-Cerrillo *et al.,* 2010, Schock *et al.,* 2001).

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).

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).

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.

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 (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 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 here described 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*
2. Evaluate different recombinant *N. caninum* stage-specifically expressed antigens for the detection of specific antibodies in cattle
3. Determine the seroprevalence of bovine neosporosis in British dairy cattle
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
5. Explore perceptions and approaches to the diagnosis and control of bovine neosporosis in current veterinary practice in the United Kingdom.

3. Materials and methods

3.1. Objective 1: Identification and expression of *N. caninum* bradyzoite-expressed antigens for ELISA development

3.1.1. 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).

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; 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.

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%).

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

3.1.3. PCR amplification

Selected sequences were amplified by PCR from *N. caninum* (NC-1 strain) DNA. 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 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

$(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 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 3.1).

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-B	F 5'-gcatgcGCAGTTTGTTTGGACGGAG-3' R 5'-aagcttGCTTTGACAGCCGACCATGAAT-3'	*Sph*I *Hind*III	58	525
NCLIV_040495-A*	F 5'-gcatgcAAGAGAATAGATGTTGTTAGCAC-3' R 5'-cccgggAGATGATCCTCTCGACAAGTC-3'	*Sph*I *Xma*I	58	606
NCLIV_040495-C	F 5'-gcatgcCACACTACCCTCGATAATGGAGAG-3' R 5'-aagcttGGCAGGGTTTCTCTGGCG-3'	*Sph*I *Hind*III	58	603

Table 3.1 – 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/**).

3.1.4. **Cloning**

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. 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. 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.

Cloning target sequences into the pQE-30 vector

QiaExpress® pQE vectors (Qiagen, Venlo, The Netherlands) 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 (Ni^{2+}).

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 3.1) 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% CO_2 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.

3.1.5. Expression of recombinant proteins

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% CO_2 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.

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-l-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 μl PBS and dyed with 83 μl NuPAGE® LDS Sample Buffer

(Invitrogen, Carlsbad, CA, USA). The residual bacterial culture was centrifuged at 2,000×*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 × 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 dH_2O on an orbital shaker, stained with Simply Blue™ Safe Stain, Coomassie® G250 stain (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour, washed with dH_2O 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 pl/Mw tool in ExPASy (http://www.web.expasy.org/compute_pi/).

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). 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×*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 NaH_2PO_4, 200mM Na_2HPO_4, 500mM NaCl) 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.

3.1.6. **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), 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) 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 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 NaH_2PO_4, 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]). 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]) 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).

3.1.7. **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 dH_2O 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. The dialysis buffer was renewed after 4 hours, changed to 2M urea dialysis buffer overnight, and finally changed to 1M urea dialysis buffer 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.

3.1.8. **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.

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.

3.1.9. **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, 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) 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 (αBovIgG-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.

3.1.10. **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).

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.

3.1.11. **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. 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.

3.1.12. **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 (αBovIgG-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 (OD_{450}).

3.2. Objectives 2 & 3: Evaluation of antibody ELISA tests and estimation of *N. caninum* seroprevalence in British dairy cattle

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**

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 (OD_{450}) were measured on a plate reader (Dynex Technologies, Chantilly, VA, USA). According to the manufacturer's instruction, results were deemed valid if the mean OD_{450} of the positive control was >0.350 and the ratio of the mean OD_{450} 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} sample – OD_{450} negative control) / (OD_{450}Dpositive control - OD_{450} negative control) × 100, where OD_{450} is the mean value of the optical density of the duplicates.

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.

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 (ggtaccGGTGTCGGGTGCGTTCAAG) 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.

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).

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.

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).

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 Paragraph 3.1.8 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 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.

3.2.4. **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 (αBovIgG-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 (OD_{450}). ELISA protocols are summarised in Table 3.2.

Controls and plasma samples were analysed in duplicate. Based on OD_{450} 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 OD_{450} of the positive controls was >0.8 and the ratio of the mean OD_{450} of the positive control duplicates and the negative control duplicates was >5.

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® *Neospora caninum* iELISA (IDVet)	Purified *N. caninum* extract (sonicated tachyzoite lysate)	Alvarez-Garcia *et al.*, 2013	-	-	1:10	-
rNcSRS2 iELISA	Recombinant NcSRS2	-	0.1	Marvel (4%)	1:100	1:2,000
rNcGRA7 iELISA	Recombinant NcGRA7	Aguado-Martinez *et al.*, 2008	0.2	BSA (2%)	1:100	1:4,000
rNcSAG4 iELISA	Recombinant NcSAG4	Aguado-Martinez *et al.*, 2008	0.1	Horse serum (2%)	1:100	1:4,000
rNcBSR4 iELISA	Recombinant NcBSR4	Jiménez-Ruiz *et al*, 2013	0.2	BSA (2%)	1:100	1:3,000
rNcSRS9 iELISA	Recombinant NcSRS9	Jiménez-Ruiz *et al*, 2013	0.2	BSA (2%)	1:100	1:3,000

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 3.1.10 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.

3.2.5. **Data analysis**

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 distribute 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 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.

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 (κ = 0.01-0.20), fair (κ = 0.21-0.40), moderate (κ = 0.41-0.60), substantial (κ = 0.61-0.80) and almost perfect (κ = 0.81-1.00) (Landis and Koch 1977). Confidence intervals were reported alongside the Kappa-values.

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 $p<0.05$.

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

3.3.1. 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 3.3). 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 cm^2 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% CO_2 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 2-5×10^7 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 3.3 – 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 *et al.,* 1988	Congenitally infected dog pup (brain)
BPA1	Bovine	USA	Conrad *et al.,* 1993	Aborted foetus (brain)
JAP-2	Bovine	Japan	Yamane *et al.*, 1998	Congenitally infected calf (brain)
JAP-4	Bovine	Japan	Yamane *et al.*, 1998	Congenitally infected calf (brain)
Nc-Beef	Bovine	USA	McAllister *et al.,* 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 *et al.*, 2000	Congenitally infected dog pup (brain and spinal cord)
Nc-LivB1	Bovine	UK	Davison *et al.,* 1999b	Stillborn calf (brain)
Nc-Poland	Bovine	Poland	Cabaj *et al.*, unpublished	Aborted foetus (brain)
Nc-Porto	Bovine	Portugal	Canada *et al.*, 2002	Aborted foetus (brain)
Nc-Spain2	Bovine	Spain	Regidor-Cerillo *et al.*, 2008	Naturally infected calf (brain)
Nc-SweB1	Bovine	Sweden	Stenlund *et al.*, 1997	Stillborn calf (brain)

3.3.2. Clinical samples

DNA samples extracted from brain, heart or placenta of 28 bovine aborted foetuses, in which *N. caninum* DNA was previously detected by ITS1 nested PCR (Katzer *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.

3.3.3. Genomic DNA extraction

Laboratory-maintained N. caninum isolates

Parasite pellets from both cultured and cryopreserved *N. caninum* tachyzoites (~2-5×10^7 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.

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 (Katzer and Bartley, unpublished).

DNA extraction was performed from tissue homogenate as outlined in Bartley *et al.* (2013a) 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.

3.3.4. **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.,* 2013a, Buxton *et al.,* 1998). PCRs were run in triplicate and the PCR products were electrophoresed on a 1.8% (w/v) agarose/TAE gel 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.

3.3.5. **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, chromosomic 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 in Table 3.4. 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 3.4 – 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 3.5).

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).

3.3.6. **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× NH_4-based Reaction Buffer, 0.75 μl 50mM $MgCl_2$, 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 μl per reaction. Approximately 0.2 μg/reaction (2 μl of the 0.1 μg/μl stock) of template or control DNA or 2 μl 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 μl/reaction) for the secondary round.

PCR products were separated by electrophoresis on 1.8% (w/v) agarose/TAE gel 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 3.5 – 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	Amplicon size
	Forward	Reverse	Forward	Reverse	(°C)	(bp)
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	TAGTACCTTGTGCCGCCAAT	TTGCTCTTCTTCCCCTGCT	*GTCTCTGTTGACCCCTCAGC	CACCTCGTCACTCCCATGTA	57	328
MRI_027	GGCCCGTCCTCTAAATGAGT	TTAAGGCCGACCCTTTACTG	*TGGCGTCTACACTGTGATCC	CGTCTGCACGTATGTCCGTA	57	369
MRI_030	TGCACACATGCATCTCGTAT	AAGGCTGACGTTAGGGAACA	*ATATTCGTGCATCCATTTCG	TCCGGGCAGATTACTGTTTT	55	273
MRI_036	TGTATGCGCTTATCTGCACCT	TGCAACGACAACACTACACG	*CTGTCGGACGCCTACACATA	AAATACCCACACGCAGCATC	56	250
MRI_037	CAATCGACATGTAGCCGTTC	GAGCGACCAATCTCGTCTTC	*TCGTGTCTCCCCCATTCTAA	AAAACGAGAGAAAGCCGTCA	56	373
MRI_040	GAAAGCCCACCTGAGGATCT	TCGACACATACGCCCATCTA	*ACTATTCGGGGGACGGTAAG	CCGACGCTTGCTACCTATTC	57	225
MRI_041	AGCCCACGCTTTTACCTACC	CCAAAGTAACACGCTTCCAA	*GCACCTCCAAACACACACAA	CACCTCGCATCACGTTCATA	58	250
MRI_042	ACACGGAAGAGAGCCTAGCA	TTCGTTCACGGACAAGACAC	*GCATTCACTTTTGTCCGTGT	AACAGGAATGCCTCCAACTG	57	242
MRI_045	CCAGTGCATTCACAGAGGTG	GAAAGACAACGAGGGACGAA	*GGCACCTGCTTACTCACGTC	AATGCTCGTCTTTCGCTCTC	57	291

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

3.3.7. 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 generate for each DNA sample tested. Unique profiles were given a multilocus genotype (MLG) number.

3.3.8. 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.

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.

3.3.9. 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. beisnoiti* 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).

3.3.10. Data analysis

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.

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.

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).

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.

3.4. Objective 5: Attitudes and perceptions of large animal veterinarians towards the diagnosis and control of bovine neosporosis

3.4.1. 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. 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.

3.4.2. 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.

3.4.3. **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 foetuses, 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/).

4. Results

4.1. Objective 1: Identification and expression of *N. caninum* bradyzoite-expressed antigens for ELISA development

4.1.1. Expression of candidate genes using the pQE-30 vector

Three newly designed gene fragments, tNcSRS44-A, tNcSRS44-C and tNcSRS12A-B 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).

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.

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). After reannotation, the predicted product of NCLIV_004430 remained unchanged: SRS-domain containing protein.

The successful expression of tNcSRS44-A (Figure 4.1 lanes 2 and 3), tNcSRS44-C (Figure 4.1 lanes 5 and 6) and tNcSRS12-B (Figure 4.1 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 4.1 lanes 2, 5 and 8) and 4 hours (Figure 4.1 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 4.1 lanes 1, 4 and 7).

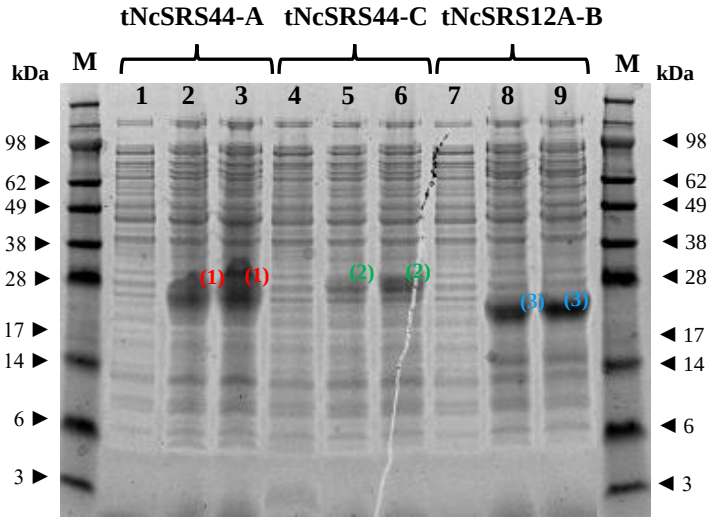


Figure 4.1 – SDS-PAGE showing protein expression of tNcSRS44-A (lanes 2 and 3), tNcSRS44-C (lanes 5 and 6), tNcSRS12A-B (lanes 8 and 9). M – molecular weight marker; lanes 1,4 and 7 – uninduced controls; lanes 2, 5 and 8 – IPTG induced (2 hours after induction); lanes 3, 6 and 9 – IPTG induced (4 hours after induction). **(1)** tNcSRS44-A (23 kDa), **(2)** tNcSRS44-C (25 kDa), **(3)** tNcSRS12A-B (19 kDa).

4.1.2. 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 4.2).

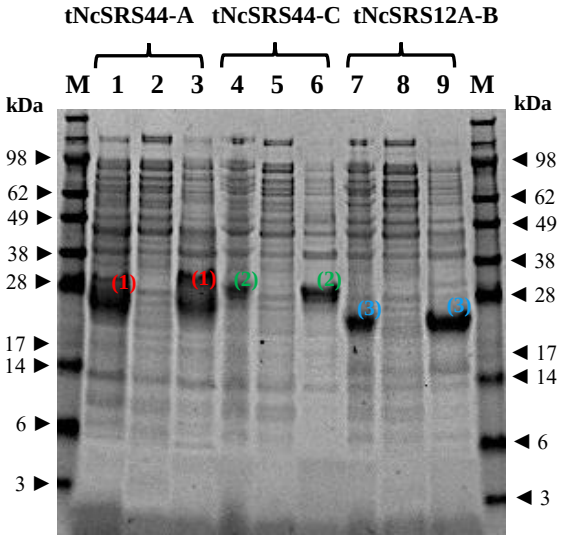


Figure 4.2 – SDS-PAGE showing the solubility/insolubility profiles of tNcSRS44-A, tNcSRS44-C and tNcSRS12A-B. All the truncated recombinant proteins were found within the insoluble fractions. M – molecular weight marker; lanes 1, 4 and 7 – induced bacterial cultures; lanes 2, 5 and 8 – soluble fractions (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).

4.1.3. **Purification of expressed proteins**

Purity of tNcSRS44-A (Figure 4.3), tNcSRS44-C and tNcSRS12A-B (Figure 4.4) 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.

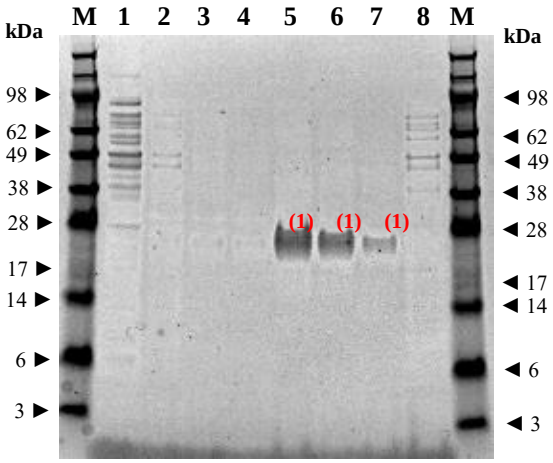


Figure 4.3 – SDS-PAGE showing purified tNcSRS44-A. Lane 1 – un-induced control; lanes 2, 3 and 4 – flow-through obtained from three sequential washes of the HisPur™ Ni-NTA Spin Columns; lanes 5, 6, 7 – flow-through obtained from three sequential elution steps; lane 8 – control supernatant before urea treatment (soluble fraction). M – molecular weight marker. **(1)** tNcSRS44-A (23 kDa).

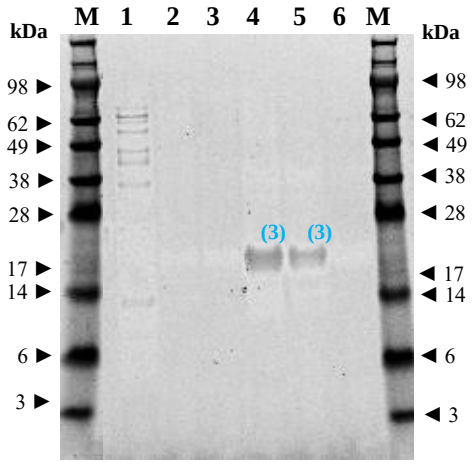


Figure 4.4 – SDS-PAGE showing purified tNcSRS12A-B. Lane 1 – un-induced control; lanes 2, 3 – flow-through obtained from two sequential washes of the HisPur™ Ni-NTA Spin Columns; lanes 4, 5 – flow-through obtained from two sequential elution steps; lane 6 – control supernatant before urea treatment (soluble fraction). M – molecular weight marker. **(3)** tNcSRS12A-B (19 kDa).

4.1.4. **Immunoreactivity assessment of recombinant proteins**

Proteins tNcSRS44-A and tNcSRS12A-B were found to be immunoreactive showing clearly visible bands in WBs (Figures 4.5, 4.6 B and 4.7 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 4.5, 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 4.6 B and 4.7 B (lanes 2) with the latter showing a slightly higher band intensity.

As shown in Figures 4.6 B and 4.7 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 4.6 A and 4.7 A) and consequently transferred to the WB (Figures 4.5, 4.6 B and 4.7 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 4.6 and 4.7). In addition, the protein bands were confirmed to be 6×His-tagged proteins as they were recognised by monoclonal anti-polyHistidine antibodies (Figures 4.6 B and 4.7 B, lanes 5).

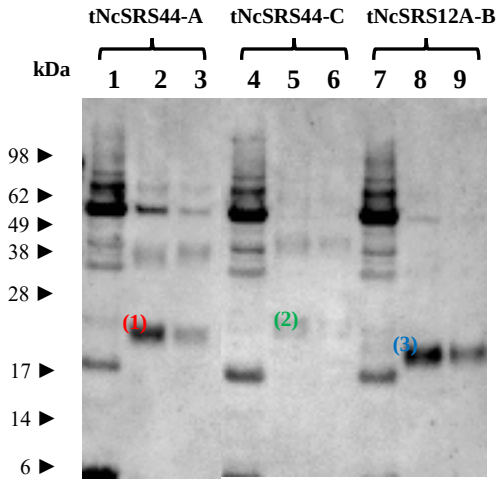


Figure 4.5 – Western blot showing the immunoreactivity of tNcSRS44-A (lanes 1, 2 and 3), tNcSRS44-C (lanes 4, 5 and 6) and tNcSRS12A-B (lanes 7, 8 and 9) when assayed with the serum of a persistently infected cow that tested negative with ELISAs based on tachyzoite antigens (bradyzoite positive sera). 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).

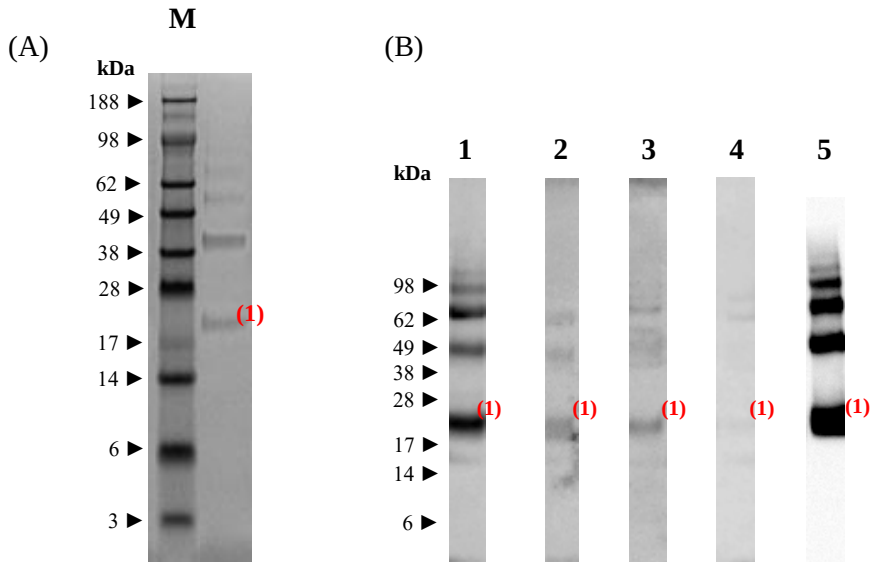


Figure 4.6 – SDS-PAGE analysis showing dialysed tNcSRS44-A (A) and Western blot based on tNcSRS44-A (B). 1 – Bradyzoite positive: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX Laboratories, Westbrook, 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).

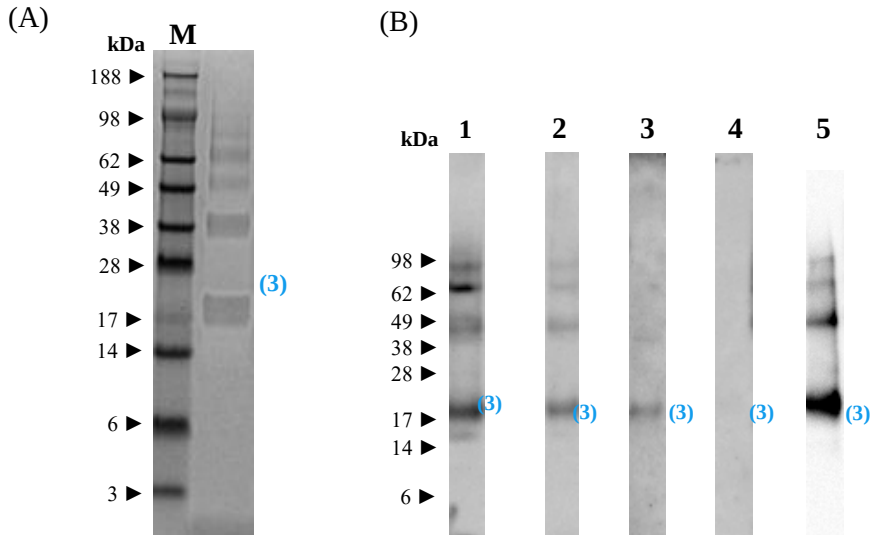


Figure 4.7 – SDS-PAGE analysis showing **dialysed tNcSRS12A-B** (A) and **Western blot based on tNcSRS12A-B** (B). 1 – Bradyzoite positive: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX Laboratories, 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).

4.1.5. **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 4.8 C).

As shown in Figure 4.8 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 4.8 A and B, lanes 1) and with the pool of four *N. caninum* antibody negative sera (Figure 4.8 A and B, lanes 2) as positive and background negative control respectively.

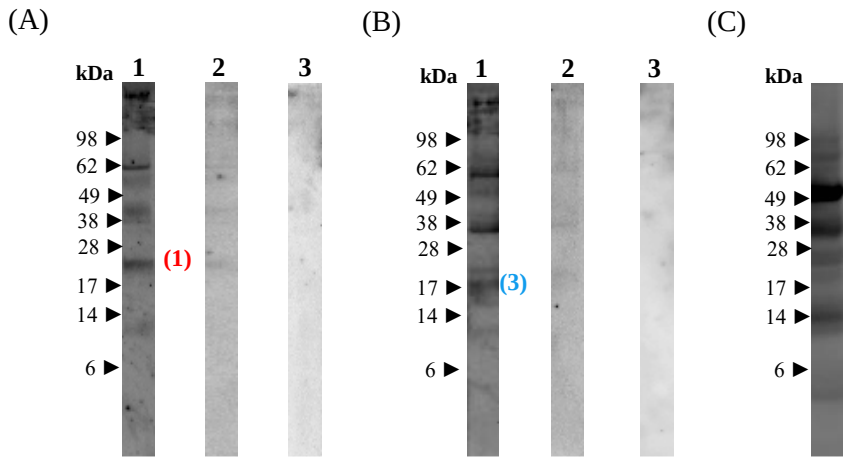


Figure 4.8 – Western blots showing the specificity of **tNcSRS44-A** (A) and **tNcSRS12A-B** (B). 1 – Positive control 1: serum from a *N. caninum* persistently infected cow that tested seronegative with current commercial serological tests (IDEXX *Neospora* Ab Test, IDEXX Laboratories, Westbrook, ME, 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.

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

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 (αBovIgG-HRP) dilutions. 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 (data not shown).

4.2. Objectives 2 & 3: Evaluation of antibody ELISA tests and estimation of *N. caninum* seroprevalence in British dairy cattle

4.2.1. 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 3.1). The geographical distribution of the herds tested is shown in Figure 4.9.

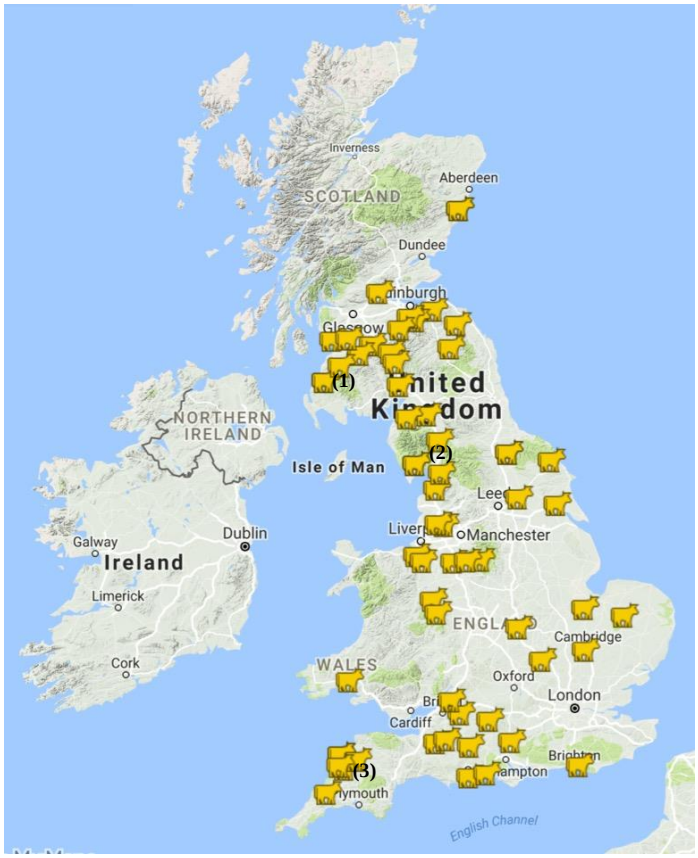


Figure 4.9 – Geographical distribution of the herds tested (*n*=61) based on the first three figures of the farm postcode (map generated using www.google.co.uk/maps). The postcodes (1) KA26, (2) LA12, (3) EX39 identify two distinct herds located within the same postcode area respectively.

Table 4.1 – 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

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

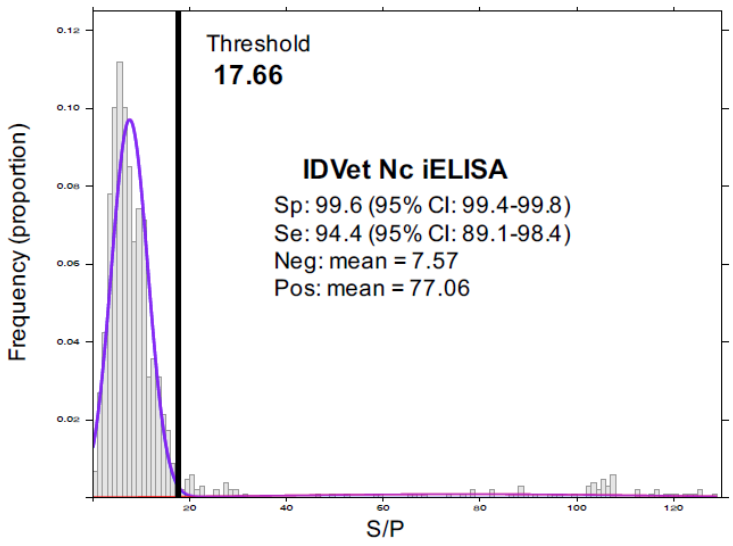
The lactation number, that is indicative of parity and age, was reported for 952 out of the 1,037 (91.8%) cows and ranged between 0 (heifers due to calve for the first time) and 10 (mean ± SD 2.67 ± 1.71, median 2.00). All heifers belonged to the “dry cows” group representing 28.0% of the animals in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

4.2.2. **Threshold determination and test characteristics**

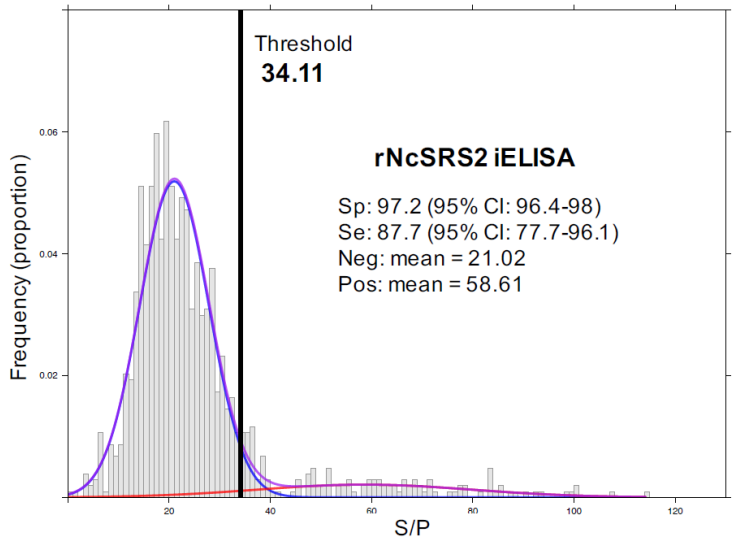
The MCMC bi-modal normal mixture model converged upon two distinct distributions with median estimates representing the mean of the negative and positive population for each of the 6 antibody ELISAs used (Figure 4.10). Optimum threshold values (defined as the highest total sensitivity and specificity 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 (Table 4.2).

Figure 4.10 – 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)



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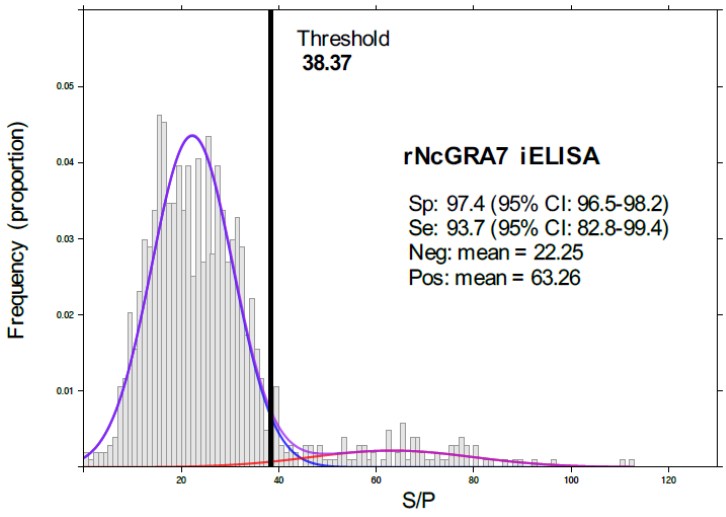
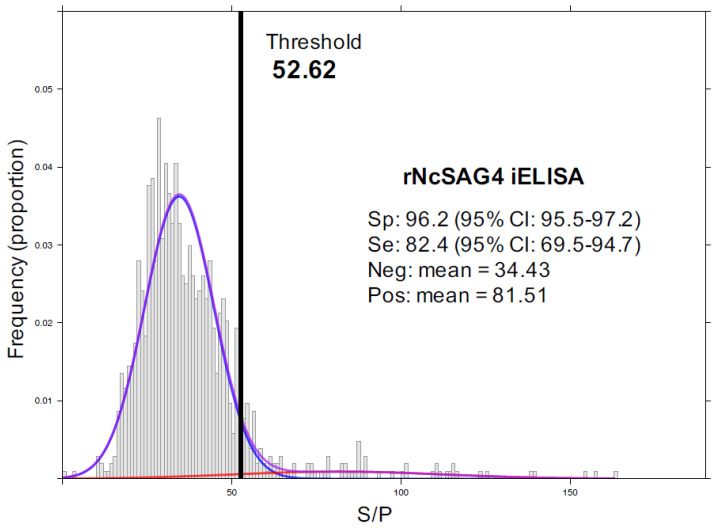
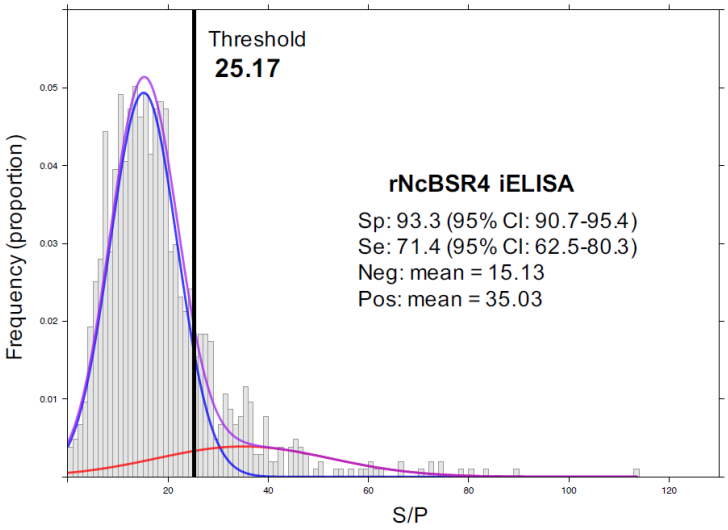
(C)



(D)



(E)



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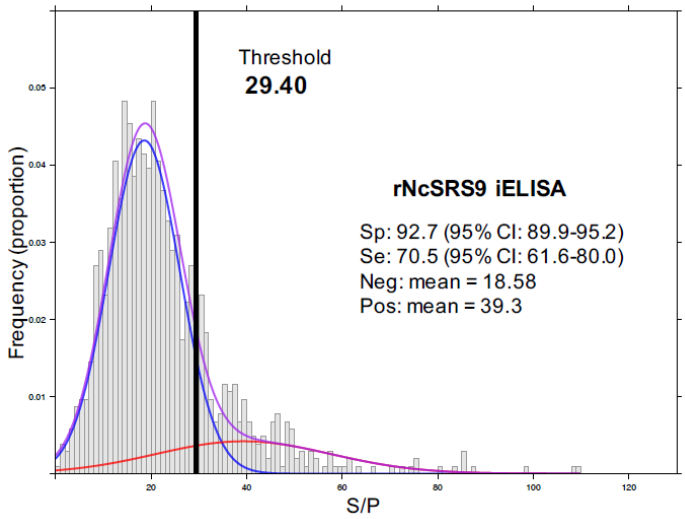
(F)



From the estimated thresholds and the distributions fitted to the observed data, the model provided median sensitivity and specificity for each test analysed (Table 4.2).

Table 4.2 – 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® *Neospora caninum* 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 4.3). 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 4.3).

Table 4.3 – 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® *Neospora caninum* 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)

4.2.3. **Test agreement**

The agreement between each combination of test pair, expressed as Kappa statistic (κ) with confidence intervals, is shown in Table 4.4. Moderate agreement (κ = 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 (κ = 0.01-0.20) with tests using bradyzoite-specific antigens (Table 4.4).

Table 4.4 – 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 4.5).

Table 4.5 – 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 4.6).

Table 4.6 – 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		

4.2.4. **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. 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 4.7). 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 4.2)

Table 4.7 – 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® *Neospora caninum* 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	**173**[1]	**864**[2]	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 4.8).

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 4.8).

Table 4.8 – 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	**173**[1]	**864**[2]	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.
[2]Reference negative: plasma samples which tested negative with all 6 tests.
* Based on the panel of reference samples generated.

4.2.5. **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 4.9).

Table 4.9 – *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® *Neospora caninum* 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; 95%CI: 19.6-47.0) seropositive cows. In one cluster, 12 cows out of 17 were classified as antibody positive (Figure 4.11).

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 4.11.

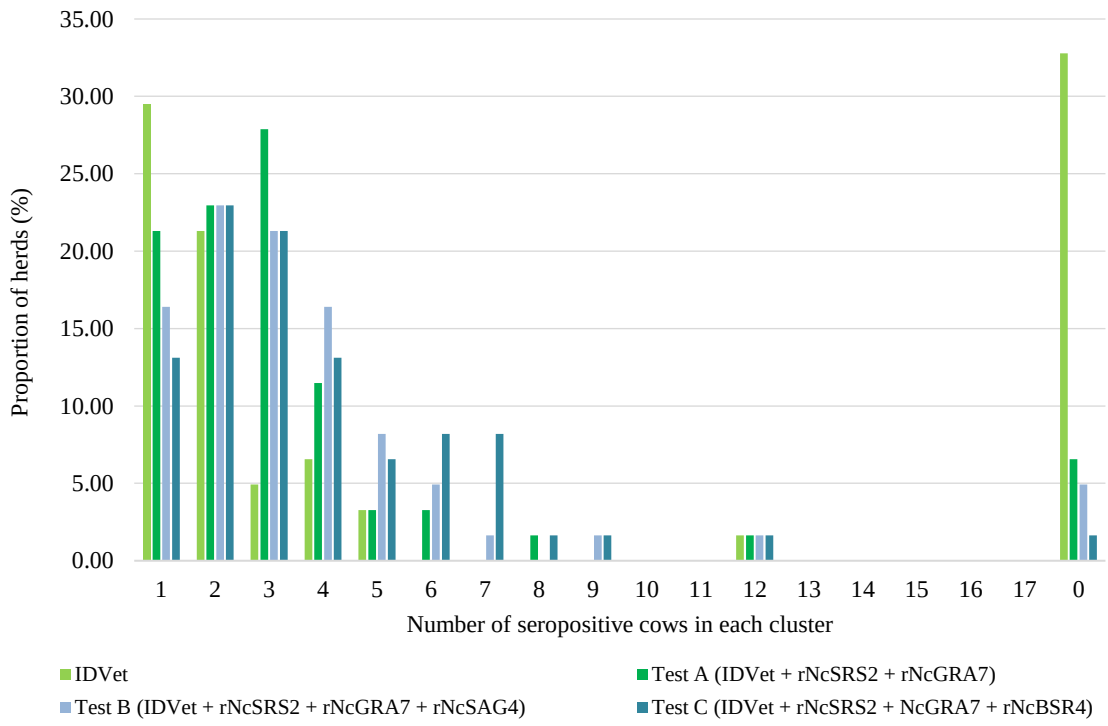


Figure 4.11 – Frequency distribution of the number of *N. caninum* seropositive cows in the sampled herds (*n*=61) as estimated with the commercial test (IDVet) and different combinations of the IDVet iELISA associated with experimental assays based on recombinant tachyzoite immunodominant and bradyzoite-specific antigens (Test A, B and C).

The observed individual seroprevalence in relation to the region of origin of the samples is shown in Table 4.10. 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 4.10).

Table 4.10 – 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 4.11).

Table 4.11 – 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.

Seroprevalence in different production groups of dairy cattle

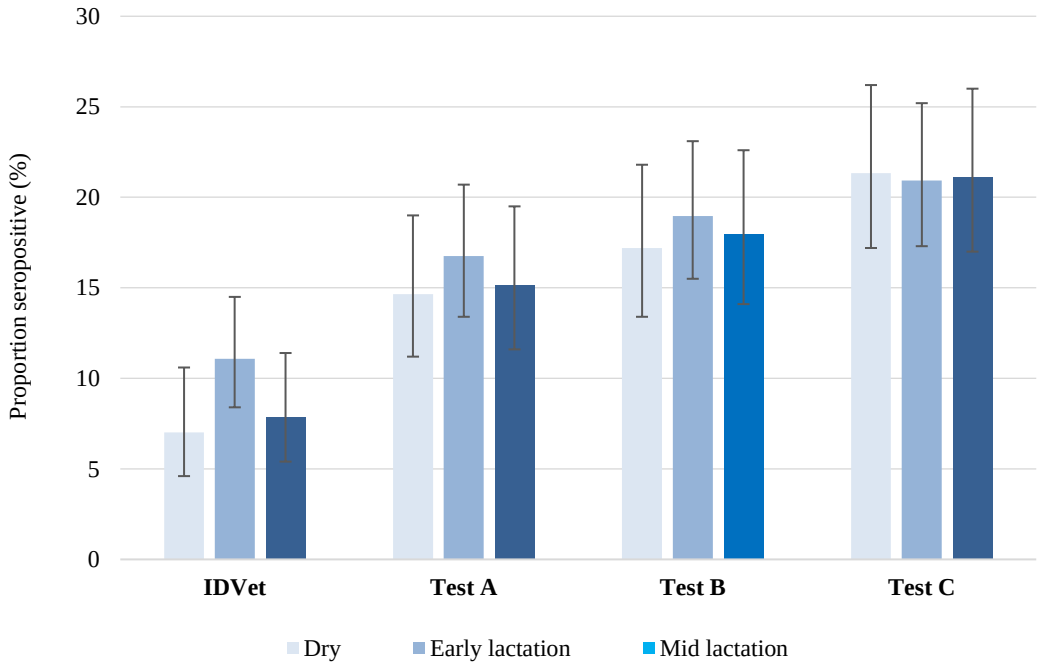
Based on the outcome of the commercial iELISA (IDVet), the observed proportions of serologically positive cattle during the dry period, in early lactation and mid-lactation were 7.0% (22/314, 95% CI: 4.7-10.4), 11.1% (45/406, 8.4-14.5) and 7.9% (25/317, 5.4-11.4) respectively (Figure 4.12). The seroprevalence 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 4.12).

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 4.12).

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 4.12).

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 4.12).



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 4.12 – 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 4.12 – 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® *Neospora caninum* 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)

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

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. 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.

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 4.13).

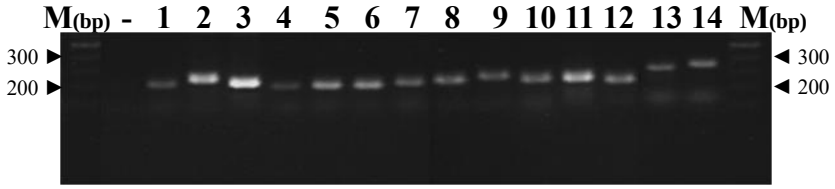


Figure 4.13 – Agarose gel showing fragment size polymorphisms of the MRI_030 microsatellite marker amongst *N. caninum* laboratory-maintained isolates. -: negative control (DNA isolated from uninfected Vero cells); 1: BPA-1, 2: Nc-Freiburg02, 3: Nc-Freiburgh03, 4: Nc-GER1, 5: Nc-Porto; 6: Nc-Spain2; 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 ≥ 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 $(AT)_n$.

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.

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 Appendix I.

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. 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. 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.

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 4.13). 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 4.13.

Table 4.13 – 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)	1.00 (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)

4.3.3. **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 4.13).

Nevertheless, all markers showed relatively high discriminatory power (SID ≥ 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 4.14).

Table 4.14 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 markers[1]	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 4.14).

4.3.4. **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 ± 0.23) (Table 4.15). A, Ar and H_{Nei} per *locus* and population are summarised in Table 4.16.

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 4.15).

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 4.15. 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 4.15 – 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 markers[1]	**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 4.16 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**

He_{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).

4.3.5. 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 4.14)

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 4.14).

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*. 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 (Appendix II).

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. 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 (Appendix II).

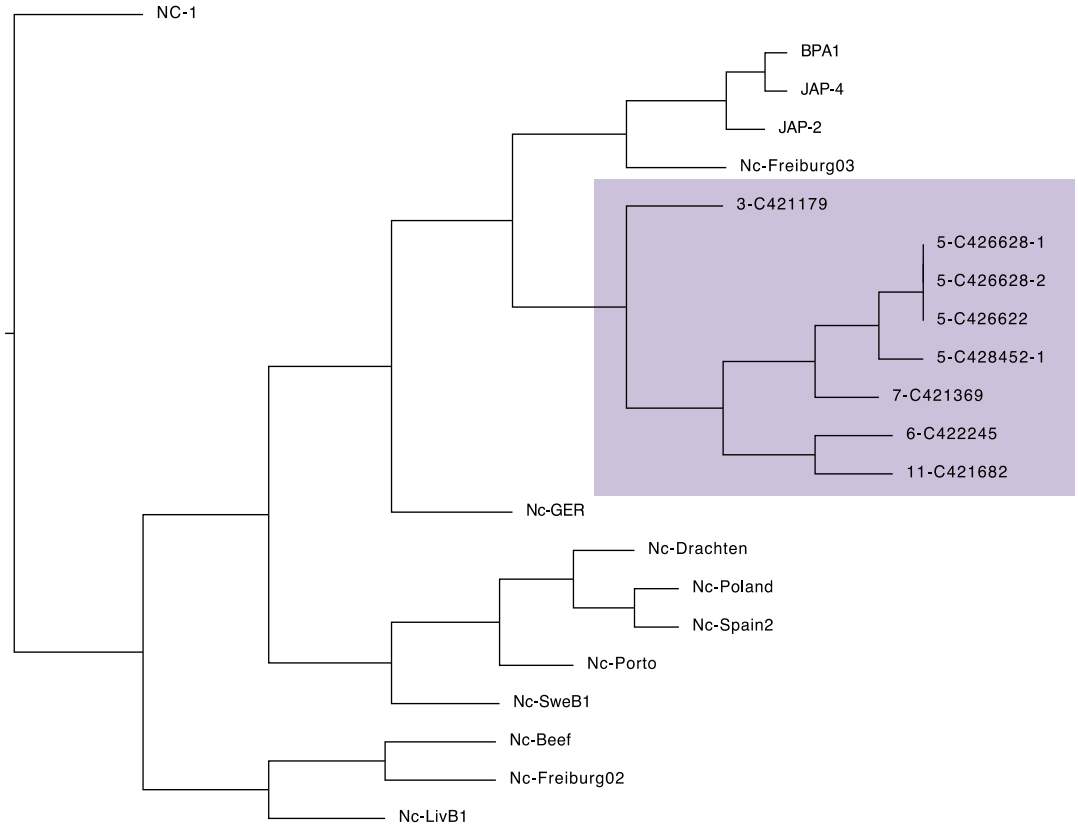


Figure 4.14 – Neighbour-joining (NJ) dendrogram showing the genetic relationships of laboratory-maintained and field *N. caninum* isolates based on multilocus analysis of the 12 polymorphic microsatellite markers investigated. Only the isolates in which an allele number was attributed in each of the 12 *loci* were included in the analysis. Clinical samples are shaded.

4.4. Objective 5: Attitudes and perceptions of large animal veterinarians towards the diagnosis and control of bovine neosporosis

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. 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 4.17).

Table 4.17 – 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

4.4.1. 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 4.15).

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 4.15).

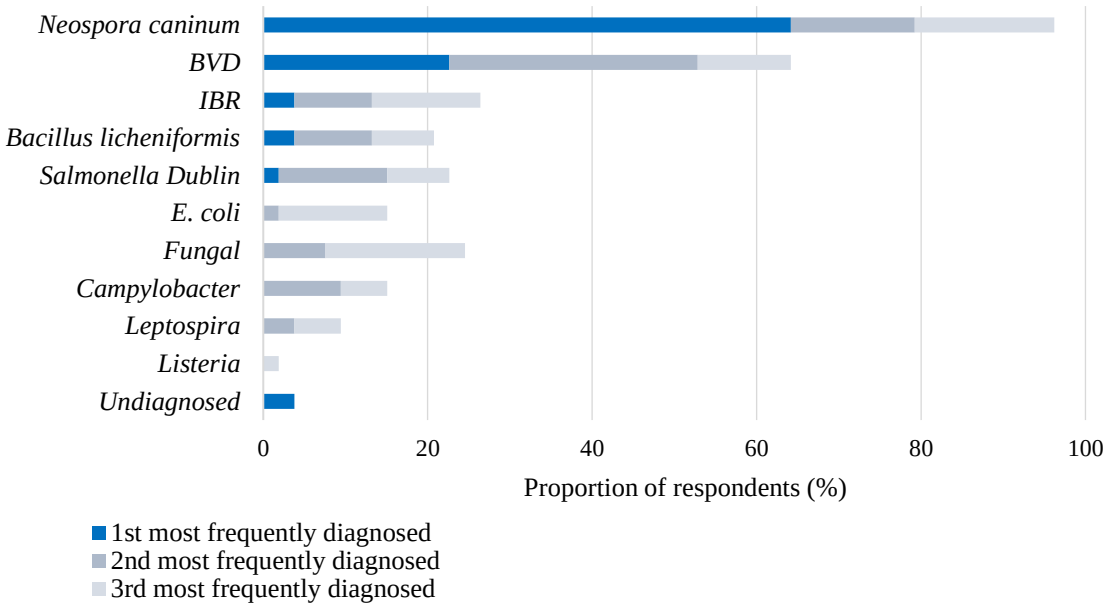


Figure 4.15 – Infectious causes of bovine abortion diagnosed most frequently according to the experience of the respondents in their geographical area of professional activity (*n*=53).

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 4.16).

Seven respondents (13.2%, 6.5-24.8) stated that all abortifacient infections were equally difficult to diagnose whilst 2 (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 4.16).

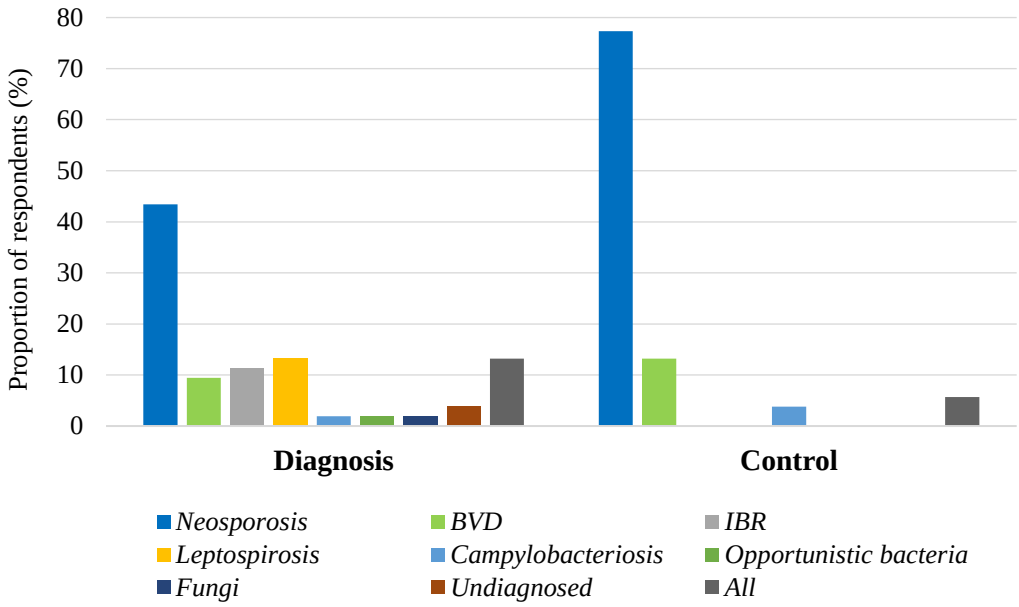


Figure 4.16 – Infectious causes of bovine abortion perceived as the most challenging in terms of diagnosis and control (*n*=53).

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 4.17).

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 4.17).

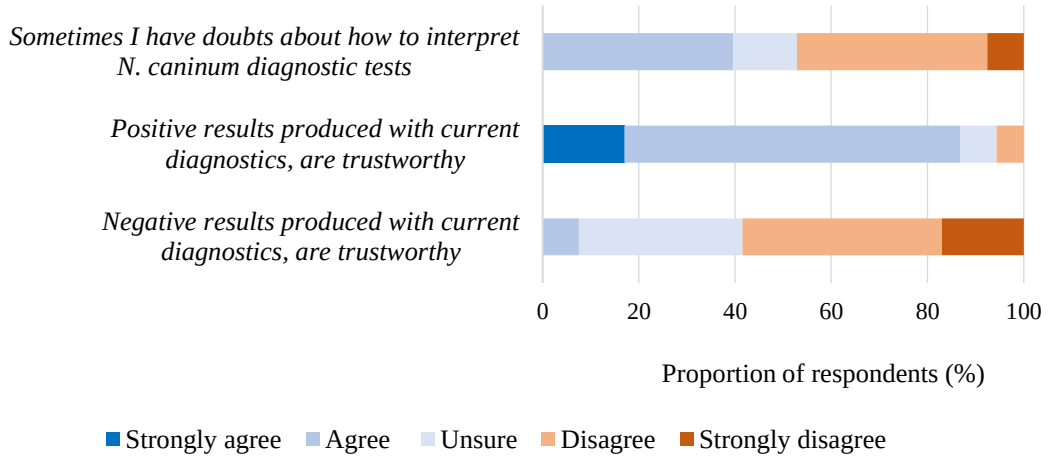


Figure 4.17 – General perceptions on the laboratory diagnosis of bovine neosporosis (*n*=53).

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 4.18). 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 4.18).

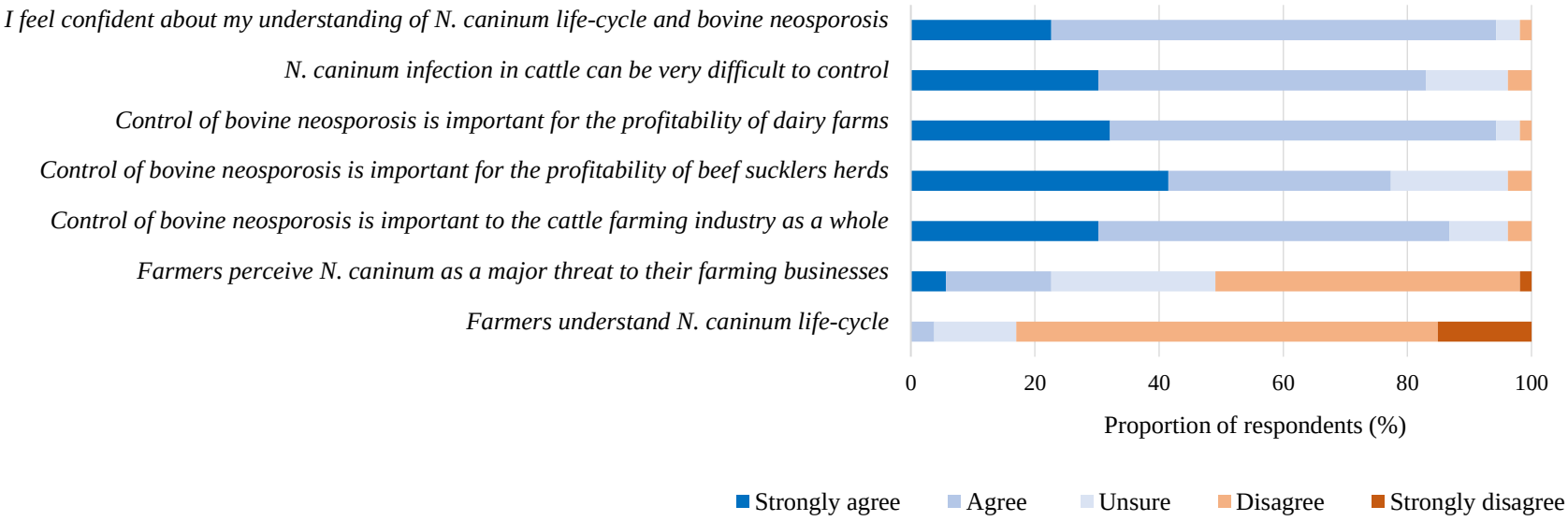


Figure 4.18 – General perceptions of *N. caninum* and bovine neosporosis (*n*=53).

4.4.2. 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 4.19). 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.

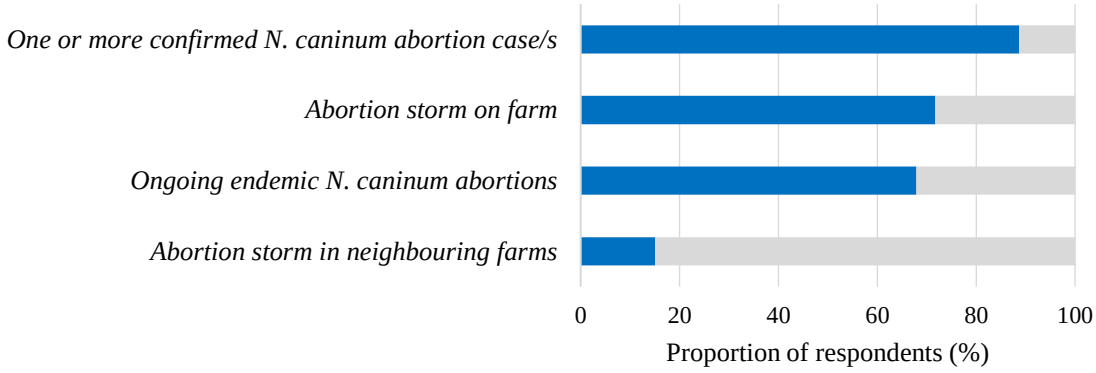


Figure 4.19 – Main triggers for the implementation of additional testing and *N. caninum*-specific control measures (*n*=53).

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 foetuses 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 4.21).

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 4.20).

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 4.20).

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 4.18. 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 4.18).

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 4.18 – Views on the reliability of current serology-based diagnostics for bovine neosporosis (*n*=53).

	Do current *N. caninum* serology-based diagnostics (ELISA) give reliable information? Why or why not?	No. Respondents	% Respondents (95%CI)
YES	*"Important consideration must be made for correct interpretation of test results (i.e. antibody titres wax and wane, limitations of available tests)"*	16	30.2 (19.5-43.5)
	"High specificity: positive results are trustworthy."	7	13.2 (19.5-43.5)
	"It is all we have got."	4	7.5 (3.9-17.9)
	"The results are consistent over time."	1	1.9 (0.3-9.4)
	"The results generally fit the clinical picture."	1	1.9 (0.3-9.4)
		29	**54.7** (41.4-67.3)
NO	*"Low sensitivity / uncertainty about negative results."*	9	17.0 (6.5-24.8)
	"Fluctuations of antibody titres."	2	3.8 (1.1-12.7)
		11	**20.8** (12.0-33.5)
Unsure		13	**24.5** (14.9-37.6)

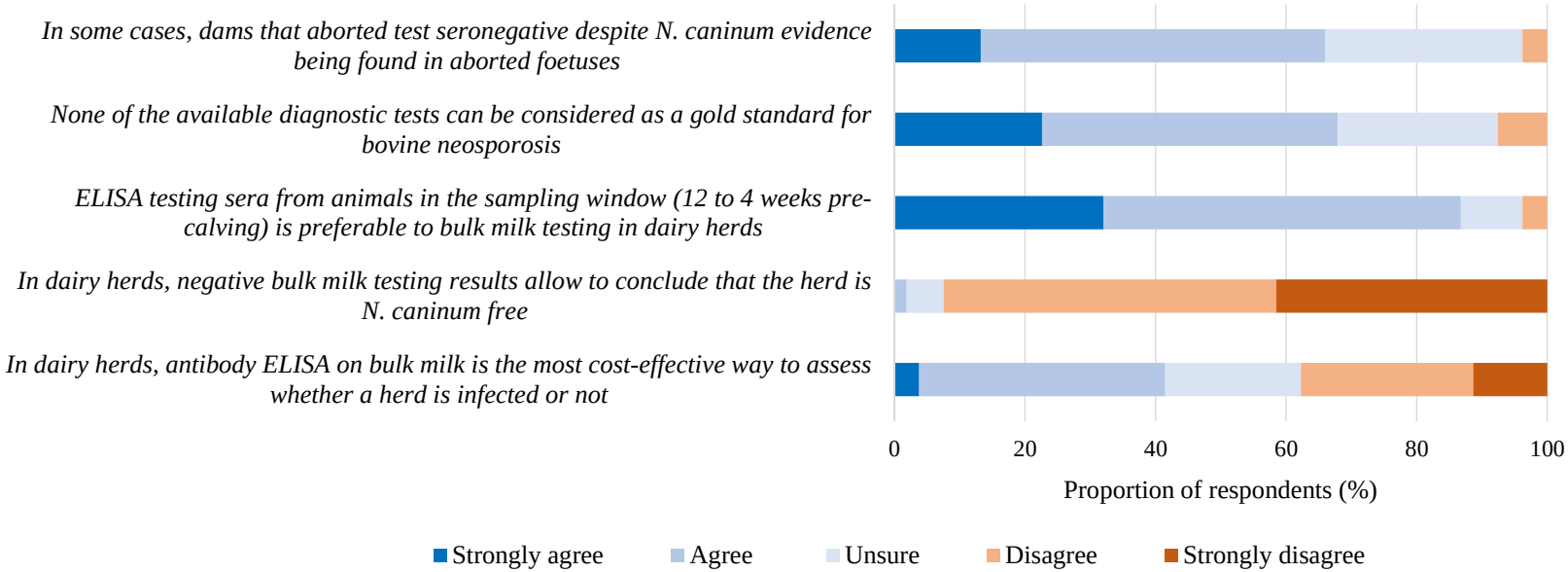


Figure 4.20 – Assessment of the presence or absence of *N. caninum* at the herd level (*n*=53)

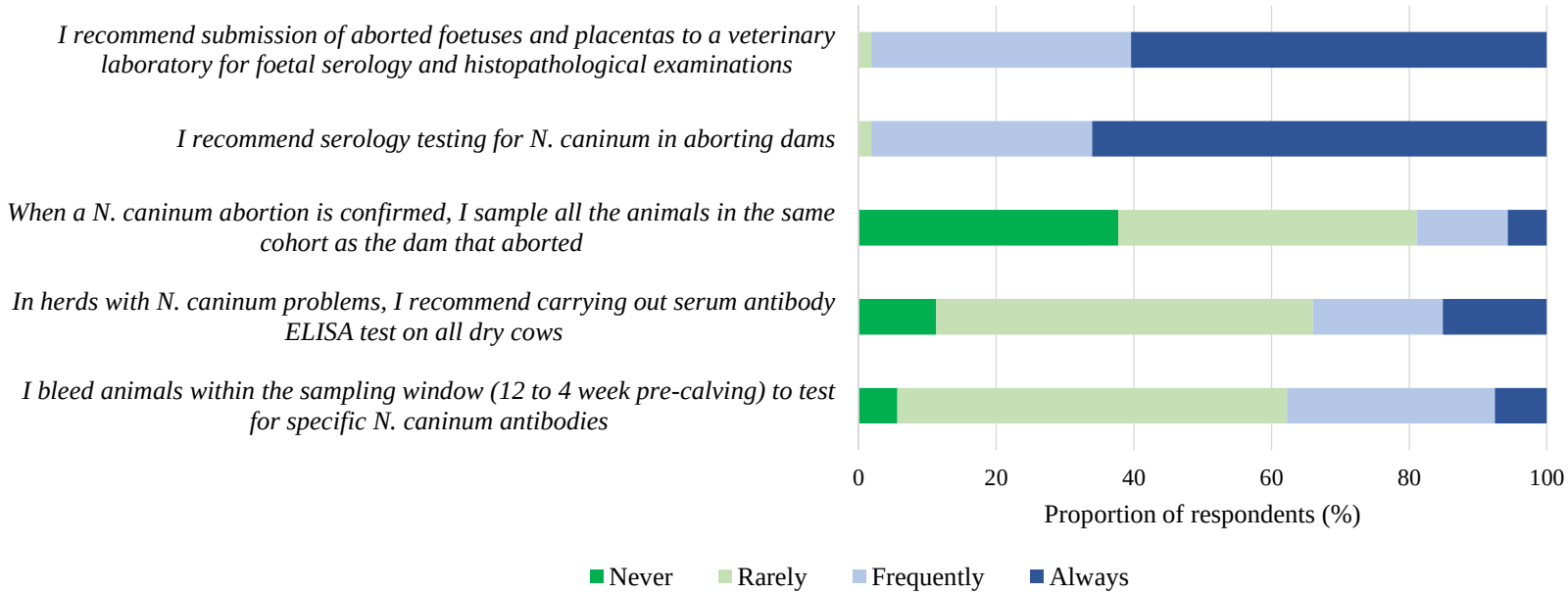


Figure 4.21 – Approaches taken during the investigation of *N. caninum* abortion outbreaks (*n*=53).

4.4.3. **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 4.22). 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 4.23).

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 4.22).

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 4.23).

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 4.23).

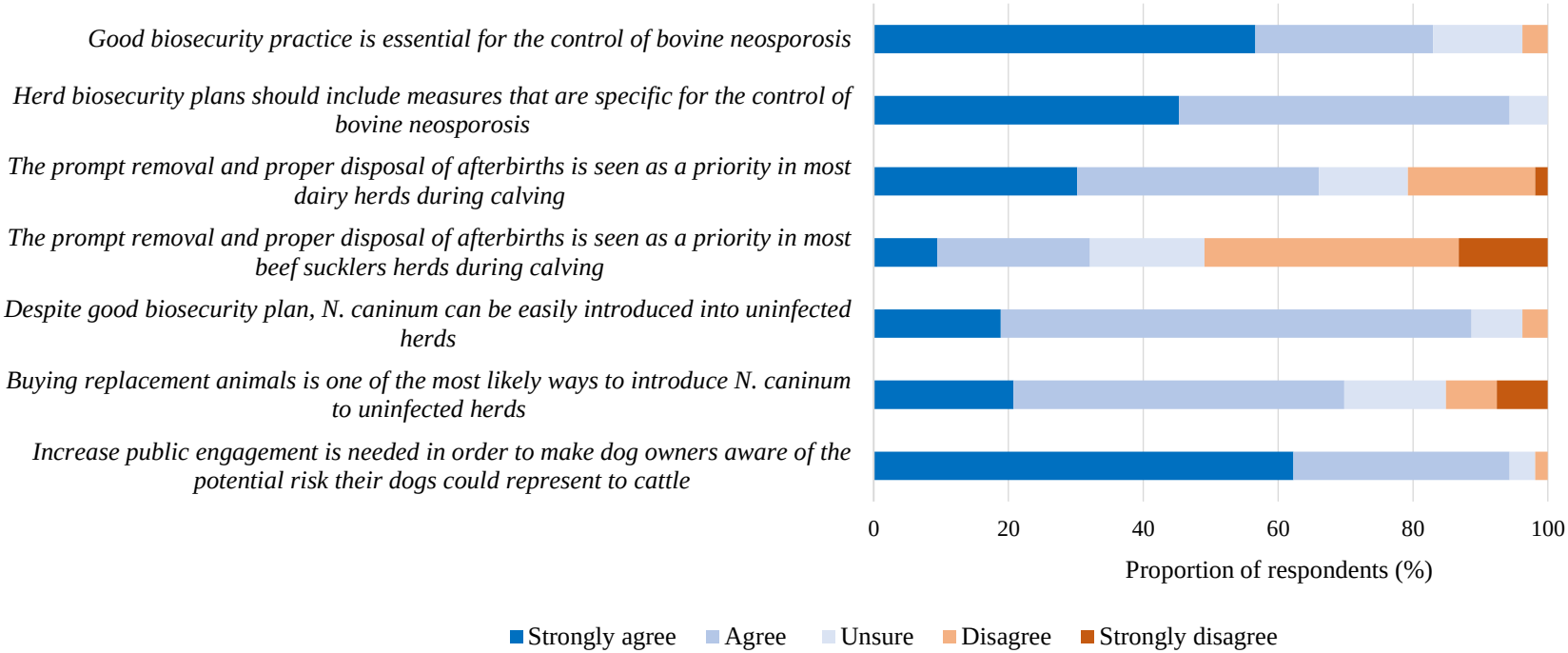


Figure 4.22 – Views on biosecurity and general hygienic measures (*n*=53)

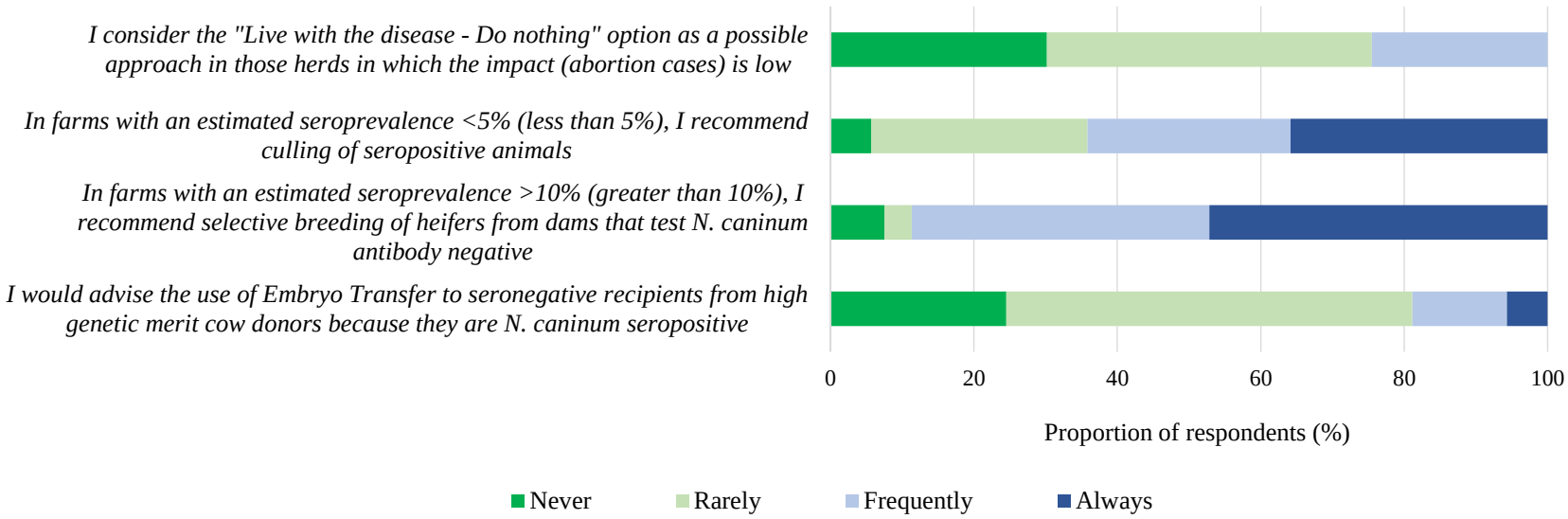


Figure 4.23 – Common practice and actions within the control of bovine neosporosis (*n*=53).

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%, 72.9-0.92) 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 4.24). 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.

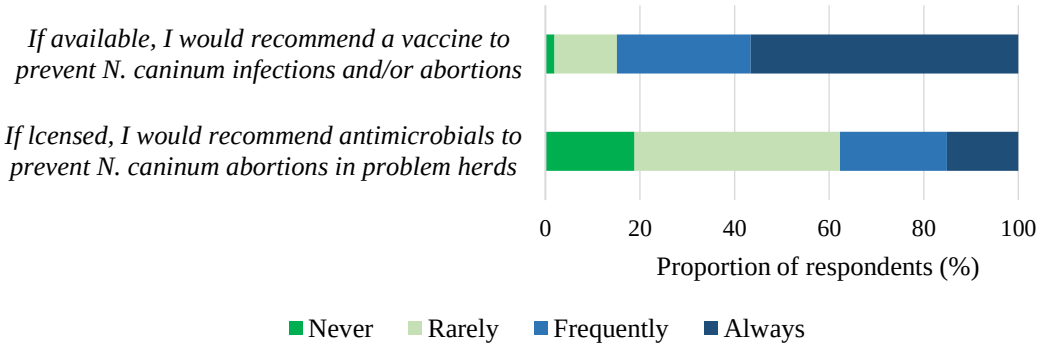


Figure 4.24 – Prospective use of vaccination and antimicrobial therapy for the control of *N. caninum* abortion (*n*=53).

5. Discussion

The detection of those animals that, despite harbouring *N. caninum*, test serologically negative with current commercially available tests represents one of the main challenges in the serological diagnosis of bovine neosporosis (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.

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.,* 2007a). In addition, recombinant proteins are easily produced in large quantities with standardised methods (Howe *et al.,* 2002).

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 (**objective 1**).

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. 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.

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 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; however, it may have interfered with the recombinant antigens when used to coat the ELISA plates.

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). The evaluation of antibody ELISA tests for the detection of *N. caninum*-infected cattle (**objective 2**) and the estimation of the seroprevalence of neosporosis in British dairy cattle (**objective 3**) were amongst the main aims of this research project.

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). 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.

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 4.8).

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 4.10 – E-F).

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 4.8). 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 4.9).

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 4.8). This suggested that further optimisation and validation of these tests are required.

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 4.9). 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.

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 4.9).

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.

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. 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 5.1).

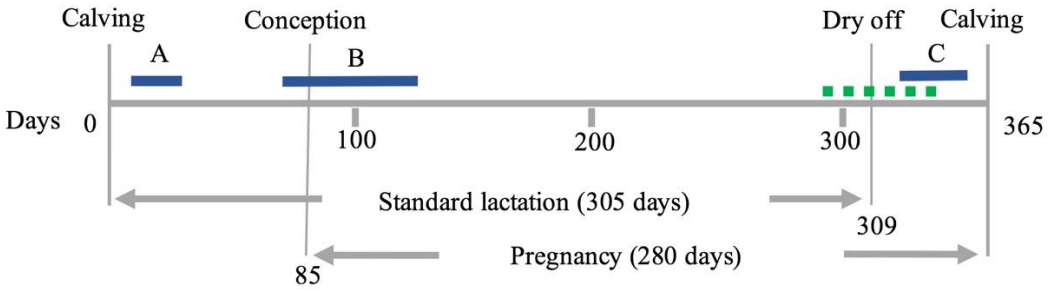


Figure 5.1 – Standard dairy cows' productive cycle with blood sampling time intervals (blue lines): (A) early lactation (B) mid-lactation and (C) dry period. The green dashed line indicates the 12 to 4 weeks pre-calving sampling window historically recommended by the main UK provider of cattle health 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 ± 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 as its minimum.

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.

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* (Katzer *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 foetuses 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).

One of the aims of the present project 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 (**objective 4**).

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 (Appendix I). 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 4.13). 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≥0.75) of the twelve markers described (Table 4.13). Within the clinical samples collected from a specific area of Scotland, the novel alleles detected at 8 out 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. 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 4.13). 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.

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. 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 4.15).

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 4.15), 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 4.14); 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). 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. 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 4.14). 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.

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.

The questionnaire-based survey for cattle veterinary practitioners (**objective 5**) showed that, consistently 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 4.15). 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 4.16). 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 4.16).

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 4.17). 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 4.17).

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 4.21).

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. 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.

6. Industry messages

Two *N. caninum* antigens expressed as recombinant proteins (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 exclusively on tachyzoite antigens. Seropositivity with the tNcSRS12A-B and tNcSRS44-A Western Blots 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.

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.

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 foetuses 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.*, 2010a), 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.

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 bovine neosporosis.

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8. Appendices

8.1. Appendix I

Allele number allocations and sizes measured by sequence analysis and automated fragment sizing (CE) for each of the 12 microsatellite markers.

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
	$(AT)_{22}$	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
	$(TATG)_{21}$	9	415	416-417
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.

8.2. Appendix II

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	
	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	
6	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