# Epidemiology of *Neospora caninum* Infection in Cattle

## Evaluation of diagnostic tests and herd studies

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**Doctoral thesis**
**Swedish University of Agricultural Sciences**
**Uppsala 2004**

**Acta Universitatis Agriculturae Sueciae**
Veterinaria 175

ISSN 1401-6257
ISBN 91 576 6670 9

Tryck: SLU Service/Repro, Uppsala 2004

# Abstract

Frössling, J. 2004. *Epidemiology of* Neospora caninum *infection in cattle. Evaluation of diagnostic tests and herd studies.*
Doctor's dissertation
ISSN 1401-6257, ISBN 91 576 6670 9

The intracellular parasite *Neospora caninum* is an important cause of abortion in cattle. The aims of this thesis were to characterize *N. caninum* dynamics in infected herds and to validate diagnostic tests for individual- and herd-level testing. The thesis is based on four separate studies.

To validate an *N. caninum* iscom ELISA, sera from 244 Swedish dairy cattle were analysed. These were also analysed by an IFAT, which was considered an imperfect reference test. Estimates of sensitivity and specificity at different cut-offs were therefore obtained using a latent class method (Gibbs sampling). The best over all cut-off was 0.20 where sensitivity and specificity were 99% and 96%, respectively. Optical densities ≤0.15 and ≥0.55 were suggested limits to rule out and rule in infection.

A three-year study of an American beef herd demonstrated how the avidity iscom ELISA is used to describe the duration of infection. The avidity pattern gradually changed as the peak shifted towards a higher avidity. It was concluded that postnatal infection was first frequent but decreased during the following years. The high avidity values at the end of the study confirmed that the majority of cows were chronically infected and that their calves were congenitally infected.

Both increased and decreased prevalences of *N. caninum* were seen when 15 Swedish dairy herds were investigated in a longitudinal study. These changes were not related to the prevalence that each herd had when first sampled. Avidity measurements and investigation of serological status of related animals revealed that most seropositive individuals were chronically infected and that vertical transmission was the dominant transmission route.

Test results from three groups of herds (total n=244) were used in an evaluation of the iscom ELISA when used to analyse bulk milk. Accuracy was estimated for different cut-off levels by using a standard cross-tabulation technique and also, in order to adjust for milk yield covariates, a logistic regression model. There was a relationship between within-herd prevalences and bulk milk optical densities. Herds with test results >0.20 are likely to be infected and herds with results <0.05 will have a within-herd prevalence of at most 10-15%.

*Keywords:* bovine, sensitivity, specificity, vertical, horizontal, transmission, prevalence, gold standard, latent class, Gibbs sampling, cut-off, genealogic trees, avidity, milk yield.

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# Shoshin

**Beginner's Mind**

# Contents

# Appendix

## Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

**I.** Frössling J, Bonnett B, Lindberg A & Björkman C. 2003. Validation of a *Neospora caninum* iscom ELISA without a gold standard. *Preventive Veterinary Medicine 57*, 141-153.

**II.** Björkman C, McAllister M, Frössling J, Näslund K, Leung F & Uggla A. 2003. Application of the *Neospora caninum* IgG avidity ELISA in assessment of reproductive losses after an outbreak of neosporosis in a herd of beef cattle. *Journal of Veterinary Diagnostic Investigation 15*, 3-7.

**III.** Frössling J, Uggla A & Björkman C. Prevalence and transmission of *Neospora caninum* within infected Swedish dairy herds. *Manuscript*.

**IV.** Frössling J, Lindberg A & Björkman C. Evaluation of an iscom ELISA used for detection of antibodies to *Neospora caninum* in bulk milk. *Manuscript submitted for publication*.

# Abbreviations

| | |
|---|---|
| BVDV | bovine virus diarrhoea virus |
| ELISA | enzyme-linked immunosorbent assay |
| IFAT | indirect fluorescent antibody test |
| IgG | immunoglobulin G |
| iscom | immunostimulating complex |
| *N. caninum* | *Neospora caninum* |
| Nc-SweB1 | a Swedish *Neospora caninum* isolate of bovine origin |
| Se | sensitivity |
| $Se_H$ | herd sensitivity |
| Sp | specificity |
| $Sp_H$ | herd specificity |
| OD | optical density |
| OIE | Office International des Epizooties |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| ROC | receiver-operating characteristic |
| TG-ROC | two graph receiver-operating characteristic |
| *T. gondii* | *Toxoplasma gondii* |

# Background

## The parasite

### *Introduction*

*Neospora caninum* is an intracellular protozoan parasite of phylum apicomplexa and class coccida (Dubey *et al.*, 2002). The parasite was first recognized in dogs with neurological disorders (Bjerkås, Mohn & Presthus, 1984; Dubey *et al.*, 1988). When previously found in histological samples it was probably mistaken for the closely related parasite *Toxoplasma gondii*. A few years later also bovine *N. caninum* infection was described (Thilsted & Dubey, 1989; Anderson *et al.*, 1991). During the 1990's several research reports have shown that the parasite is an important cause of bovine abortion in many parts of the world and that both acutely and chronically infected cattle have an increased risk to abort. Description of the pathogenesis and clinical effects of *N. caninum* infection, as well as the parasite's structure and biology, and the host-parasite relationship, are reviewed in Dubey and Lindsay (1996) and Dubey (2003).

### *Definitive hosts*

The relation to *T. gondii* led researchers to suspect that *N. caninum* had a similar life cycle – with intermediate hosts and a carnivore definitive host. Before the complete life cycle of the parasite was known, findings in epidemiological studies indicated that the presence of dogs on a farm increased the risk of *N. caninum* abortion in cattle (Paré *et al.*, 1998; Wouda *et al.*, 1999). In 1998 experimentally infected dogs were shown to excrete *N. caninum* oocysts with their faeces and it was soon confirmed that this also took place under natural conditions (McAllister *et al.*, 1998; Lindsay, Dubey & Duncan, 1999; Gondim, Gao & McAllister, 2002). Until recently the dog was the only species verified to be a definitive host of the parasite. However, Gondim *et al.* (2004) showed that coyotes (*Canis latrans*) represent an additional definitive host. Exposure to coyotes or grey foxes (*Urocyon cinereoargenteus*) was also identified as a risk factor for *N. caninum* infection in a previous epidemiological study (Barling *et al.*, 2000). It is assumed that there may be other definitive hosts in wildlife and some common species have been investigated (Dubey, 1999; Dubey, 2003). For example, foxes have been shown to carry the parasite (Schares *et al.*, 2001) but it has not been verified that foxes shed oocysts (Schares *et al.*, 2002), i.e. are actual definitive hosts. In Belgium and Spain antibodies to *N. caninum* were demonstrated in >17% and 11% of samples from foxes (Buxton *et al.*, 1997; Almeria *et al.*, 2002), while no test-positive samples were found when Swedish red foxes were investigated (Jakubek *et al.*, 2001).

### *Intermediate hosts*

Although the consequences of *N. caninum* infection presently seem to be of highest importance in cattle, several other species, such as deer, horses and water buffalo, are also possible intermediate hosts of *N. caninum* (Dubey, 2003) and could potentially experience negative effects as a result of infection. Other possible hosts of interest are, of course, humans. Unlike toxoplasmosis, *N. caninum* infection has not been demonstrated in humans. No clearly positive samples have been found when sera from farmers, aborting women or other categories of humans have been examined for presence of antibodies (Nam, Kang & Choi, 1998; Petersen *et al.*, 1999; Graham *et al.*, 1999; Tranas *et al.*, 1999; Hemphill & Gottstein, 2000; Dubey, 2003). Moskwa *et al.* (2003) recently reported findings of *N. caninum* DNA in milk from infected cows. If it can be confirmed that the source is live tachyzoites, this would increase the urgency for further research on *N. caninum* infection in humans.

### *Transmission of the parasite in cattle*

Cattle can be infected by *N. caninum* by transmission of the parasite from the cow to her fetus during gestation, or via a definitive host. The optimal study design for investigating the degree of congenital infection is to obtain pairs of precolostral samples of the newborn calf and its mother. For obvious reasons these kind of studies are rare, but those that have been performed have shown that vertical transmission occur in 68-95% of gestations (Paré, Thurmond & Hietala, 1996; Wouda, Moen & Schukken, 1998; Davison, Otter & Trees, 1999). Thus, the majority of calves born from infected cows are congenitally infected. There are also studies that have used more indirect measures of vertical transmission, e.g. by comparing serological status of mother and offspring later in life. In these comparisons the probability of a seropositive offspring due to vertical transmission was 0-86% (Bergeron *et al.*, 2000; Dijkstra *et al.*, 2001; Romero & Frankena, 2003). The reason behind the wide range of these estimates is that they depend not just on the degree of congenital infection, but also on the presence of horizontal transmission and the time passed since the parasite was introduced into the herd.

In contrast to vertical transmission, the degree of horizontal transmission certainly differs between populations and over time. In this context, horizontal transmission refers to cattle being infected by ingestion of oocysts excreted by a definitive host. Other types of postnatal infection have been investigated but none has been shown to occur naturally. Uggla *et al.* (1998) successfully infected newborn calves by feeding them colostrum spiked with tachyzoites. The same kind of experimental infection was successfully repeated Davison *et al.* (2001). This group also found that none of the calves that were fed colostrum from infected cows were infected, and consequently concluded that this route of transmission is not of major importance. In addition, the parasite has never been shown to occur naturally in colostrum. Other researchers have discussed that ingestion of placenta or amniotic fluid expelled by infected cows could result in horizontal transmission between cattle (Ho *et al.*, 1996; Bergeron *et al.*, 2001). This has so far not been verified (Davison *et al.*, 2001).

### *Antibody response*

According to current knowledge infected individuals remain carriers of the parasite for life. It is also assumed that all infected cattle continue to produce specific antibodies. However, fluctuations in antibody levels have been observed in both experimentally and naturally infected cattle. In particular during gestation, the interaction between the host's immune system and the parasite seems to be able to cause rapid peaks and drops in detectable antibody levels. On occasion this even falls below the cut-off levels of the commonly used serological assays (Stenlund *et al.*, 1999; Guy *et al.*, 2001; Sager *et al.*, 2001; Maley *et al.*, 2001; Trees *et al.*, 2002).

Calves that are fed colostrum from infected cows obtain maternal antibodies to *N. caninum*. Hietala and Thurmond (1999) showed that, after one month, such passively acquired antibodies could still be demonstrated in 50% of sera from non-infected calves. However, in most of the calves antibodies were not detected after 2 months.

### *Tests for the demonstration of* N. caninum *antibodies*

Several assays have been developed for the demonstration of antibodies in serum. These serological tests include indirect-fluorescent antibody tests (IFAT), enzyme-linked immunoassays (ELISA), immunoblotting and *N. caninum*-agglutination tests (reviewed by Björkman and Uggla (1999). Some of these test procedures have also been evaluated for analysis of milk (Björkman, Holmdahl & Uggla, 1997; Schares *et al.*, 2004). Bulk milk tests for the demonstration of other bovine infections have been increasingly used (Niskanen, 1993; Sargeant *et al.*, 1997; Kramps *et al.*, 1999; Nylin, Stroger & Ronsholt, 2000; Beaudeau *et al.*, 2001). To date the iscom ELISA is one of two tests described for the demonstration of *N. caninum* antibodies in bulk milk samples (Björkman *et al.*, 2000; Chanlun *et al.*, 2002; Schares *et al.*, 2003).

### *Tests for measurements of avidity*

The antibodies produced at the beginning of *N. caninum* infection have a lower affinity (binding strength) than antibodies produced later on. Serological tests can be modified to measure the difference in functional affinity, i.e. avidity. In human medicine, such avidity measurements are used as complement to other tests (Hedman & Seppala, 1988; Hedman *et al.*, 1989; Zotti *et al.*, 2004) but only a few avidity tests have been developed for use in veterinary medicine. An iscom ELISA was the first test described to measure avidity of *N. caninum* IgG antibodies (Björkman *et al.*, 1999) and one of the very first described for use in the veterinary field. Subsequently, other *N. caninum* avidity ELISAs were presented (Maley *et al.*, 2001; Schares *et al.*, 2002; Sager *et al.*, 2003).

### *Tests for the demonstration of* N. caninum *antigen*

In order to diagnose *N. caninum* infection direct demonstration of the parasite is desirable. However, suitable samples are not as easily obtained as serum. Most of these methods are also not even applicable for diagnosis in live animals. Exceptions are certain body fluids (like colostrum, cerebrospinal or amniotic fluids) or tissue biopsies from placenta that can be collected from living animals. Examples of techniques for direct demonstration of *N. caninum* currently in use are histology and immunohistochemistry (Wouda *et al.*, 1997), PCR techniques (Holmdahl & Mattsson, 1996; Sager *et al.*, 2001) and isolation of the parasite (Stenlund *et al.*, 1997).

### *Isolation of the parasite*

The most definitive evidence of *N. caninum* infection is isolation of the parasite. This can be carried out in cell culture directly from post-mortem samples, or via inoculated mice. Unfortunately, isolation of the parasite from infected tissues is not always successful. However, in spite of many failed attempts, a certain number of isolates from different parts of the world has now been obtained (Dubey *et al.*, 2002; Dubey, 2003). The NC-SweB1 isolate was one of the first bovine isolates and is the only so far from Sweden (Stenlund *et al.*, 1997). Laboratory stocks of NC-SweB1 tachyzoites have been shown to differ in certain aspects when compared to other isolates. On comparison it seems like the Swedish isolate is less virulent and has a slower propagation rate *in vitro* (Atkinson *et al.*, 1999; Schock *et al.*, 2001). Analysis of genetic diversity has demonstrated some differences between *N. caninum* isolates but has not shown that NC-SweB1 diverges more than others (Atkinson *et al.*, 1999; Schock *et al.*, 2001). In fact, Schock *et al.* (2001) reported no genetic clustering of isolates with respect either to host or geographical origin.

## Evaluation of tests

To correctly interpret the results of a diagnostic test it is essential that its performance has been appropriately evaluated. All tests will produce some false results and therefore it is not realistic to blindly trust results of any diagnostic test. Much is gained however if the type and magnitude of the misclassifications that can be expected is estimated. General guidelines on how to validate diagnostic tests have been given by the OIE in the Manual of standards for diagnostic tests and vaccines (http://www.oie.int/eng/normes/mmanual/A_summry.htm; 27-Apr-2004).

### *Measures of consistency and accuracy*

Firstly, a diagnostic test should meet the basic demands of consistency (or precision). This refers to the ability of the test to produce the same result on different runs, days, batches etcetera. In other words, variation within the laboratory should be low (which equals high repeatability). Ideally, the test should also have a high consistency between different laboratories (i.e. high

reproducibility). This is however not as critical as the internal consistency, as long as differences are described and taken into consideration when results from different laboratories are compared. However, for tests used e.g. in connection with international trade, there is also a need for a high consistency between laboratories.
In addition to being consistent, results of a diagnostic test need to be accurate. Traditionally accuracy is measured by calculating the sensitivity (Se) and the specificity (Sp) of the test (Rothman & Greenland, 1998). Se is the proportion of test positive results in the diseased (or infected) subgroup of the population. Sp, on the other hand, is the proportion of test negative results in the non-diseased (or non-infected) subgroup. (Note that these definitions refer to the epidemiological Se and Sp, which are different from the analytical sensitivity and specificity (Saah & Hoover, 1997).) For tests measured on a continuous scale, these two measures of test performance are inversely related so that as one increases, the other decreases. Assays for direct detection of agents constitute an exception as these may have Sp=1 that is not affected by changes in Se.

It has been shown that test accuracy may depend on the composition of the population tested. Examples of factors that can influence test performance are age, sex, breed, gestation month, infection load and duration of infection (Courtney & Cornell, 1990; Sockett, Carr & Collins, 1992; Greiner & Gardner, 2000; Lindberg *et al.*, 2001). This may be reflected in the apparent prevalence of a disease (or infection) – in spite of the fact that by definition Se and Sp are not affected by changes in prevalence. It is therefore important to evaluate the test for the population in which it will be applied, and that the estimates of accuracy are based on a representative sample of that population. When there are other factors that have impact on test accuracy, estimating Se and Sp for the relevant population strata is preferable (Greiner & Gardner, 2000). As the composition of a population changes with time, for example during an eradication program, test performance should ideally be repeatedly updated
(OIE, http://www.oie.int/eng/normes/mmanual/A_summry.htm; 27-Apr-2004).

### *Dealing with imperfect reference tests*

One of the problems with measures of accuracy is that the traditional way of evaluating the performance of a test relies on having a gold standard, i.e. an independent method that correctly identifies each animal's true disease status (Thrusfield, 1997). True gold standards are rare and therefore imperfect tests have often been used as the reference (Dohoo, Martin & Stryhn, 2003). The consequence of using an imperfect gold standard is that the estimates of Se and Sp will be biased as correct results of new and perhaps superior tests may be interpreted as misclassifications. There are different ways to handle this problem. Multiple testing (serial or parallel) has been used to improve classification of true disease status (Fletcher, Fletcher & Wagner, 1996; Dohoo, Martin & Stryhn, 2003). Confirmed cases or populations with known disease status, such as SPF herds can also be used for reference (Thrusfield, 1997). However, these are often not representative of the actual target population. If there are estimates of the Se and Sp of the reference test, the Se and Sp of the new test can be calculated while

adjusting for the imperfectness of the reference test (Staquet *et al.*, 1981). More recently methods that do not require that Se and Sp of the reference test are known have been increasingly applied (for a review see Enøe, Georgiadis and Johnson, 2000). These so-called latent class models are based on either frequentistic (maximum likelihood) or Bayesian statistics.

### *Selection of suitable cut-off*

With tests that have a continuous outcome, a cut-off (i.e. the level above which the test result is to be regarded as positive) can be selected. Se and Sp obviously depend on this decision threshold and can vary greatly with different cut-offs (Fletcher, Fletcher & Wagner, 1996). A traditional way to select a cut-off is by analysing a sample of the healthy members of the population and to use their mean result plus two (or three) standard deviations as decision threshold (Jacobson, 1998). This method ensures ~97% Sp but disregards the level of Se. In order to make a more informed decision about the best cut-off alternative methods have been developed. Plotting a receiver-operating characteristic (ROC) curve (Hilden, 1991; Zweig & Campbell, 1993), a two graph receiver-operating characteristic (TG-ROC) curve (Greiner, Sohr & Gobel, 1995), or calculating and plotting the misclassification-cost term (Robertson, Zweig & van Steirteghem, 1983; Vizard, Anderson & Gasser, 1990; Greiner, 1996), are some examples of different methods used to choose the best cut-off for different applications.

### *Herd level testing*

To facilitate sampling of a population of animals and to reduce sampling costs, herd level testing can be applied. Sometimes the herd level result is also of primary interest, rather than individual test results – e.g. when aiming at certifying herd status in research investigations or control programs. Herd level testing can be based on pooled samples (e.g. bulk milk samples) or on interpretations from the aggregate of individual test results from each herd. Both approaches depend on the individual-level Se and Sp of the test but have different influence on the herd sensitivity ($Se_H$) and herd specificity ($Sp_H$).

$Se_H$ and $Sp_H$ based on pooled samples depends on prevalence, the number of animals in the pool (m) and the numbers of samples from the herd (r) (Christensen & Gardner, 2000). Increasing m and r increases $Se_H$ and decreases $Sp_H$. Pooled Se will often be lower than the individual Se (especially when prevalence of infection is low and m is large). Because of dilution the pooled Sp, on the other hand, is often higher than the individual Sp (Christensen & Gardner, 2000). The statements above assumes that all animals contribute equal amounts and that all contributions have the same concentration of the analyte. However, these prerequisites do not hold in the case of bulk milk samples. On the contrary, each cow's relative contribution to the pool, and the concentration of the analyte in each contribution, can be expected to vary to a great extent. The number of animals included in the pool is not optional to the investigator. Instead, pool size varies between samplings and is often large.

When an aggregate of individual test results is translated to a herd-level result, $Se_H$ and $Sp_H$ depend on the within-herd prevalence, the number of individuals tested within the herd and the minimum number of reactors determining whether a herd should be considered positive (Martin, Shoukri & Thorburn, 1992). In this case $Se_H$ increases with prevalence of infection and the number of individuals tested. A decrease in individual Sp (which results in relatively more false positive results) causes an increase in $Se_H$ and a decrease in $Sp_H$ (Dohoo, Martin & Stryhn, 2003).

# Aims

The overall goal of this thesis project was to further characterize *N. caninum* dynamics in infected Swedish dairy herds. This required validation of diagnostic tests for bovine *N. caninum* infection in order to improve interpretation of test results for various applications, including both individual samples and herd testing. Validated tests could then be applied to characterize herd status and to examine transmission patterns.

More specifically, the aims were:

- To determine the sensitivity and the specificity of the immunostimulating complex (iscom) ELISA for demonstration of antibodies to *N. caninum* in serum, and to establish optimal cut-off levels for different applications (I).

- To investigate the performance of the avidity iscom ELISA when used to describe duration of *N. caninum* infection in a herd (II).

- To evaluate the iscom ELISA for detection of antibodies in bulk milk for different cut-off levels. Further, to investigate the relationship between test results and within-herd *N. caninum* prevalences (IV).

- To use serological data from *N. caninum* infected herds to investigate how the within-herd prevalence develops over time and assess the relative importance of different routes of transmission (II, III).

# Methodological considerations

Detailed descriptions of material and methods used are given separately in each paper (I-IV).

## Study populations

Swedish dairy herds that had experienced abortion problems and been diagnosed as being *N. caninum* infected constituted the study population, or one of the study populations, in Papers I, III and IV. The herds were located in various parts of Sweden. Blood samples from all female individuals >3 months were collected from these herds. In addition, a sample from the milk tank was occasionally collected (Paper IV). Paper I was based on samples taken in five of the herds in 1999 and 2000, Paper III on repeated samplings of all of these herds (n=15) on different occasions between 1994 and 2003, and Paper IV on samples taken in of nine of the herds in 2003.

The study presented in Paper IV also involved two other groups of herds. One of these groups was primarily sampled for bulk milk and comprised 115 herds that were located in an area in the southwest of Sweden and that participated in a research project about health and growth rate in heifers. The other group was 120 randomly selected herds from a larger geographical region in southern Sweden. Blood samples from all cows as well as bulk milk samples were collected from these herds.

Paper II dealt with a beef herd in Nebraska, USA. This herd had experienced an outbreak of *N. caninum* abortion in 1998. Blood samples were collected from the cows, replacement heifers, and sometimes calves, twice yearly from autumn 1998 to spring 2001.

Most of the Swedish herds studied were enrolled in the milk recording scheme and information about age, breed and milk yield were obtained from the database of the Swedish Dairy Association. Reproduction data from the American herd were based on the farmer's recordings.

## Diagnostic tests

The diagnostic test investigated and applied in all papers were versions of the *N. caninum* iscom ELISA. In Paper I, this test was validated for analysis of serum, and in Paper IV it was evaluated for analysis of bulk milk. The serum and milk test versions were basically the same, except that sera were diluted 1:100 while milk samples were diluted 1:2. The iscom ELISA has also been modified to measure the avidity of antibodies and the avidity test method was applied in Papers II and III, together with the basic serum version.

Besides different ELISAs, the IFAT is a commonly used test procedure used for the analysis of sera for *N. caninum* antibodies. It was used as a reference test in Paper I. Furthermore, results from Western Blot analysis of dog sera are presented in Paper III.

## Methods used to investigate test performance

### *Latent class model (I)*

Since there is no perfect reference test for the demonstration of *N. caninum* infection, a latent class approach was chosen for the validation of the iscom ELISA (Paper I). The method used was the Gibbs sampler where the Se and Sp of two tests can be estimated without considering any of the tests a gold standard (Enøe *et al.*, 2001). The tests used were the iscom ELISA and the IFAT and the dichotomised test results were summarized in 2 x 2 tables – one table for each of two subgroups of the study population. In Bayesian estimation, existing information is used to define prior probabilities. As the Gibbs sampler is based on Bayesian statistics, inclusion of prior probabilities in the calculations was thus required. In the statistical procedure, posterior probabilities are calculated considering both the observed data and the prior probabilities (Gelman *et al.*, 1995). In Paper I the estimation was made using both informative and non-informative prior distributions. In an iterative process values of the parameters were randomly selected from the prior distributions and combined with estimates from data, resulting in posterior distributions for each parameter. The final point estimates of Se and Sp corresponded to the median of the posterior distributions. A credibility interval was given by the values at the 2.5 and 97.5 percentiles of the distributions.

### *Logistic regression model (IV)*

Before the evaluation in Paper IV, the iscom ELISA had been used to analyse bulk milk samples for many years, but there had been no thorough investigation on which to base selection of decision thresholds. Information about test performance, especially when applied on herds with unknown infection status, was also lacking. The aim of our evaluation was to examine if bulk milk samples could replace for individual sampling to some extent. The association between the prevalence of test-positive individuals within a herd and the bulk milk test result was also investigated.

A bulk milk sample is a special type of pooled sample; the pool size is often large and there is an obvious and substantial variation in the proportion of the sample contributed from each individual. In our study, a logistic regression model was used to estimate Se and Sp while accounting for this variation. Thus, when building a model for this purpose we were not primarily searching for factors that had impact on test performance, but rather trying to control for associations we already knew existed.

The way the composition of the bulk milk in the tank was taken into consideration was by using a set of variables summarising the milk yield of groups of cows with different antibody levels. To calculate the milk yield of each cow at the particular day of sampling, the change in milk yield between the two closest milk recordings was calculated. The milk yield at the particular day of sampling was then extrapolated from this, unless the cow was reported dry.

*TG-ROC (I, IV)*

For both test evaluations (I, IV) Se and Sp were estimated for several cut-off levels. The estimated results were presented and interpreted in TG-ROC graphs, i.e. Se and Sp were plotted for each decision threshold and in the same graph. This method offers an overview of test performance and facilitates defining critical cut-off levels where a test shows certain characteristics (Greiner, Sohr & Gobel, 1995).

## Cow-calf pairs and genealogical trees (II, III)

Vertical and horizontal transmission were assessed by comparing the serological status of cows and their calves. Although this is an apparently simple and straightforward method, the comparison can be performed in many different ways. Due to differences in preconditions, the estimates from comparison of the pairs cannot always be directly compared. The variants used in Paper II and III were slightly different. In Paper II, only the calves that were born during the last season, and their respective mothers, were considered in the investigation. In Paper III, all pairs of mothers and their offspring present at the sampling were considered. However, when analysing data from the second sampling and onwards, only pairs that were new since the last sampling were considered. By this modification, it was possible to assess events that took place between the samplings. When all cow-calf pairs of a cross-sectional sampling are compared, the presence of a group of test-positive calves with test-negative mothers does not necessarily mean that horizontal spread has recently occurred.

In Paper III the relationship between the cattle within each herd were mapped in genealogical trees. These were supplemented with all test results available for each individual. The trees were used not only to determine the nature of transmission but also to make assessments about certain individuals' true infection status.

# Results and discussion

## Changes in cut-off – changes in accuracy (I)

In Paper I it was established that when using the optical density (OD) value 0.20 as cut-off, the *N. caninum* iscom ELISA had high Se and Sp (98 and 96%, respectively). Further, the changes in accuracy for the whole range of decision thresholds, as visualized in a TG-ROC, provided valuable information about how to interpret test results on all levels. A 0.55 cut-off resulted in high Sp (99%) and was suggested as limit to rule in infection. Correspondingly, a 0.15 cut-off, where Se was high (99%), was chosen as the limit to rule out infection. It should be remembered that these decision thresholds are approximations and not fixed values. Because accuracy varies with the dynamics of the population tested, the decision thresholds must also be changed to fit the current conditions. The results showed that the iscom ELISA is a useful and flexible tool for diagnosis of *N. caninum* infection.

Although in reality there are only two alternatives – either the individual is infected or it is not – some valuable information is lost when the antibody test results are dichotomised. In ELISAs, the odds of an individual being infected usually increase with increasing test values. These odds are sometimes presented as likelihood ratios, which give a quantitative expression of test performance, basically summarizing the same type of information that the probabilities of Se and Sp give (Fletcher, Fletcher & Wagner, 1996). There was clearly an increased probability of *N. caninum* infection with increasing iscom ELISA OD values. This correlation between OD value and probability of infection was however not direct to the extent that it would be biologically relevant to only use the continuous results. Continuous values are also not practical when results are to be presented to people outside the laboratory, e.g. clinicians or farmers. The best compromise between a single cut-off and continuous results is to use several decision thresholds. By using the cut-off levels discussed in Paper I, tested individuals can be divided in five groups with different probabilities of infection. This kind of grouping constitutes a good foundation for conclusions to be used in clinical work, control schemes or in research studies as performed in Paper III and IV.

## Using the Gibbs sampler (I)

One feature of this work was that the TG-ROC plot was based on estimates obtained by a latent class method. When the wide range of decision thresholds in the ELISA was used, the test performance varied as could be expected. The point estimates of Se varied between 18 and 99%, while Sp varied between 45 and 99%. In spite of this extreme variation in ELISA performance, the point estimates of IFAT were relatively stable. Although credible intervals were sometimes wide, the point estimates of IFAT Se and Sp varied between 72-88% and 96-100%, respectively. This illustrated the robustness of the Gibbs sampler. If the estimates for one of the tests were completely insensitive to the performance of the other test, there would have been no variation at all in the IFAT Se and Sp. It is worth

noting that by retrieving Gibbs sampler results for a wide range of cut-offs in one of the two tests, while keeping the cut-off in the other test – and consequently the performance – unchanged, information about the performance of the Gibbs sampler was gained.

### *Assumptions behind estimates of accuracy*

With the Gibbs sampler the investigated tests are assumed to be independent. Although there are methods to test for conditional dependence between tests (Enøe et al., 2000), the model that was used in Paper I does not allow such testing. If dependence between tests was present, error rates would be underestimated for both tests (Enøe et al., 2000). Using more traditional methods for estimating accuracy would not eliminate the risk of such underestimates because they also depend on the assumption that the compared tests are independent.

The other assumption required for Gibbs sampler calculations is that Se and Sp for both tests are the same for both subgroups (Enøe, Georgiadis & Johnson, 2000). We had no particular reason to suspect that this assumption did not hold. Fluctuations in antibody levels during gestation and indications of increased risk of false positive results for certain age groups (Hietala & Thurmond, 1999) have been reported. However, both subgroups had individuals of different age and gestation status and there were no indication of overrepresentation of certain groups of animals. Another factor that has been said to affect accuracy is duration of disease (Greiner & Gardner, 2000). Regarding *N. caninum* infection, the research results in this matter are ambiguous. For example, in one study *N. caninum* positive cows from herds with epidemic abortions had lower ELISA values than *N. caninum* positive cows from herds with endemic abortions (Schares *et al.*, 1999). On the other hand, results from another investigation showed that aborting cows had higher ELISA values and that newly infected cows were the ones more likely to abort (McAllister *et al.*, 2000). Further, to date we have no information that Se or Sp differs for horizontally infected compared to vertically infected animals. After the first few weeks post infection, a *N. caninum* positive animal's level of antibodies is regarded as a poor indicator of the recency of infection.

### *Including prior knowledge*

As this latent class method is based on Bayesian statistics, inclusion of prior knowledge was allowed. Such priors may be based on previous work and/or expert knowledge. Some may argue that this increases the risk of introducing subjectivity into the analysis. However, when used in parallel with non-informative priors the risk of a concealed incorrect influence of the prior information should be low. Thus, non-informative priors were and should be used to evaluate the impact of prior information (Enøe, Georgiadis & Johnson, 2000). The uniform distribution is not a realistic guess for a test's Se and Sp but serves as a conservative estimate. Although the priors can be subjective or based on incorrect evaluations, these "guesstimates" are often close enough to represent the true values. Obviously, it is important that the researchers judge whether their final

estimates are reasonable. In our case informative priors made little difference and consequently neither improved nor harmed estimation. The results confirmed our impression of the iscom ELISA being the more sensitive and the IFAT being the more specific of the two tests. Before the validation study was performed there were many years of experience of using the iscom ELISA in research and diagnostic work. The IFAT is sometimes used in serial testing to verify borderline ELISA results. From this it was suspected that the ELISA was more sensitive and less specific than the IFAT. The estimated sensitivity of the IFAT was a bit lower than reported elsewhere (Paré et al., 1995; Packham et al., 1998; Atkinson et al., 2000). Our results also confirm that although IFAT is a well-established test that has been used for many years, it cannot be treated as a true gold standard. In comparison with the IFAT, many tests incorrectly could seem to have low specificity.

Although the assumptions of these no-gold standard methods should not be deliberately violated, uncertainty about them is in itself no reason to immediately dismiss the use of these methods. All available methods for test evaluation have different strengths and weaknesses in estimating test accuracy. There is an ongoing improvement in methods for validation of diagnostic tests. All together, different estimation procedures provide information that contributes to the understanding of test performance. In the current context, using these methods is better than just relying on methods that are known to be incorrect and that rely on similar assumptions.

## Bulk milk testing (IV)

Besides the serological application, the iscom ELISA test system can also be used to detect antibodies in bulk milk samples. In Paper IV, with similar objectives as in Paper I, a cut-off investigation was done to learn more about test accuracy and possible usage of the bulk milk test. By using the suggested cut-offs it can be judged whether a herd is infected or not. As the bulk milk test result depends on the number of infected cows, the actual OD value is perhaps more informative when testing bulk milk than when testing individual sera. The results in Paper IV showed that the OD values were related to the within-herd prevalence.

By the investigation of changes in accuracy with changing cut-offs in Paper IV, decision thresholds with high Sp or high Se could be selected. It was shown that at a 0.20 cut-off the point estimates of Sp were ~99% and under practical conditions, this can be used as a limit to rule out infection. Some infected herds were found to have a very low bulk milk OD, and thus even a low cut-off (0.05) did not ensure 100% Se. However, below this level, herds are less likely to be infected and will have a within-herd prevalence of at most 10-15%. Also, with repeated sampling as in a monitoring programme, an increase in within-herd prevalence would soon be detected.

The bulk milk sample is a type of pooled sample that more or less makes 100% Se impossible. By bulk milk sampling it is not possible to control for each cow's production level, lactation phase or the number of cows included in the pool. The results of the logistic regression model confirmed that the bulk milk test

performance is affected by the respective milk production of infected and non-infected cows. However, with this in mind, the test can still be useful. By repeated sampling there is a good chance of demonstrating antibodies from infected cows even though the milk production for each cow varies with lactation month. In practice, bulk milk testing has been proven to be of value in surveillance and control of various bovine infections (Lindberg & Alenius, 1999; Nylin, Stroger & Ronsholt, 2000). One way to control for the milk production of different cows, or other factors that may affect test performance, is to use spot tests. In other words, instead of just picking one single sample from the milk tank, samples of the same volume from a predefined number of individuals with similar characteristics can be collected and pooled (for examples see Lindberg & Alenius, 1999). It is known that *N. caninum* serum antibody levels fluctuate during gestation (Stenlund *et al.*, 1999) and if it would be described that peaks or drops in milk antibody levels are seen in particular gestation months, cows could in theory be grouped according to this factor, thereby increasing the probability of detecting the infection.

Three different classification criteria were defined to compare the bulk milk test result to the aggregate of individual serum results. These were based on the number of positive animals at three levels of cut-off discussed in Paper I. Using standard methods for calculating herd-level test performance based on individual test results (Martin et al., 1992), it can be seen that false positive herds are a general problem when prevalence, as in this case, is low. In particular for the classification criteria based on the lowest cut-off, a substantial part of herds classified as positives could be expected to be false positives. Consequently, comparing the bulk milk test to this classification criteria probably underestimated bulk milk Se. In addition, the low prevalence in the sampled populations in Paper IV did not permit a precise estimate of Se. However, almost 100% of the herds that were negative based on serum results were almost certainly true negatives and therefore it is expected that the Sp estimate for the bulk milk test is fairly accurate for this population of herds.

## Investigation of routes of transmission (II, III)

Knowledge about the infection pattern and modes of transmission within a herd is necessary in order to formulate useful and appropriate recommendations to the farmer. To examine signs of horizontal transmission is also of value for research purposes, especially when studying sources of infection and in the search for definitive hosts. In addition to the demonstration of seroconversion in individuals, studies of cow-offspring pairs, genealogical trees and avidity patterns have been useful to reach an understanding of the routes of transmission.

### *Avidity*

Paper II clearly illustrates how the avidity iscom ELISA can be used to investigate the duration of the infection. The avidity pattern gradually changed as the peak shifted towards a higher avidity. During the abortion outbreak in 1998 (McAllister *et al.*, 2000), the mode of avidity was 21-40, and stabilized at 61-80 two years later. The mean avidity increased from 30.1 in 1998 to 73.7 in 2001. A similar

development in avidity pattern was seen in the results from the calves of each year. Absence of cows and calves with high avidities at the first sampling indicated that the within-herd prevalence was low before the outbreak. It was concluded that postnatal infection was frequent at the time of the abortion outbreak. However, during the following years, the rate of horizontal transmission decreased. The high avidity values at the end of the study confirmed that the majority of cows were chronically infected and that their calves were congenitally infected as a result of vertical transmission.

In Paper III, avidities were measured in eleven of the herds. In eight of these herds, 90-100% of test-positive sera had avidities >40. In other words, the majority of the individuals were chronically infected. The information provided by avidity results in Paper II and III was in agreement with the information from the other serological results and comparison of serological status of related individuals.

### *Serological status of related animals*

In Paper II there was an association between serological status of the mother and offspring, which was not seen during the preceding abortion outbreak (McAllister *et al.*, 2000). Vertical transmission was thus considered to be the dominant route of transmission and was estimated to occur in 85% of calvings by seropositive dams. Several dams (22%) had seropositive offspring, indicating that there was also a high rate of horizontal transmission during the study period. In order not to overestimate the degree of vertical transmission or underestimate horizontal transmission rate, the numbers of this comparison were adjusted. The reasoning behind this was that positive mothers also might have been postnatally infected. Similar adjustments have been performed by Romero and Frankena (2003). This kind of adjustment can be recommended when horizontal transmission is present. In the mother-offspring comparison in Paper III such adjustments were not made since indications of horizontal transmission in these herds were very rare. Regardless of adjustments, comparing pairs of cows and their offspring from a cross-sectional study is not an optimal method to estimate the degree of congenital infection in case horizontal transmission is present. However these comparisons can contribute to the understanding of the relative importance of the different routes of transmission.

As each cow-calf comparison in Paper II only considered calves born during the latest season, and their respective mothers, the current degree of postnatal infection could be assessed. When, on contrast, all cow-calf pairs of a single cross-sectional sampling are compared (Dyer *et al.*, 2000; Dijkstra *et al.*, 2001; Romero & Frankena, 2003), the presence of a group of test-positive calves with test-negative mothers does not necessarily mean that horizontal spread has recently occurred. However, by using results from two (or more) samplings (Paper III), it was possible to assess events that took place between the samplings. For cows that have more than one calf present in the herd, misclassification of her serostatus may have a relatively larger impact on estimates. To modify for this, Dyer *et al.* (2000) randomly selected one calf per cow to be considered in comparisons. We

chose to consider all available pairs in order to make use of valuable information that otherwise would be lost.

When the results of all cow-calf pairs in the herds of Paper III were summarized, 86% of calves from seropositive cows appeared to be postnatally infected. Some seronegative cows also had positive offspring (14%). These were not evenly distributed in time or among herds, but mostly present on a few occasions in three of the herds. Low OD values and information from repeated samplings indicated that a large proportion of seropositive calves from seronegative cows were false positives. Supplementing family lines with all antibody test results obtained for each individual allowed simultaneous browsing of pedigree data and repeated measures. By this mapping it was possible to assess both how and when the parasite had been spread in the herd. From the genealogic trees it was shown that the infection had been congenitally transferred for at least 5-6 generations in some herds.

By summarizing assessments from Paper III (i.e. repeated samplings of individuals, comparison of serological status of related individuals, and the herd avidity ELISA results), it was concluded that vertical transmission was by far the dominant route of transmission in all herds studied. It was also confirmed that even if horizontal transmission is rare, vertical transmission is sufficient for the parasite to silently be passed on for many generations and sustain a certain level of within-herd prevalence.

## The *N. caninum* situation in Sweden

### *Prevalence in regions and within infected herds (III, IV)*

The sampling of the study populations in Paper IV provided two estimates of herd-level seroprevalence of *N. caninum*. Both estimates indicated that the herd prevalence in the investigated part of Sweden was at least 6%. There is no previous herd-level estimate of the *N. caninum* prevalence in Sweden. The within-herd prevalences in herds where test-positive cows were identified varied between 1 and 36%.

There was a large variation in within-herd *N. caninum* prevalence (5.8-65.0%) when the herds of Paper III were first sampled. Both increased and decreased within-herd prevalences were seen over the years, and they were not related to the level of prevalence that each herd had when first sampled. Thus, even a low within-herd prevalence constitutes a potential risk for further spread of the infection in a herd. Three herds were free or almost free from infection at their last sampling.

### *Why is prevalence low and horizontal spread rare?*

From the results in Paper III it can be concluded that horizontal spread is relatively rare in Sweden. The estimate from previous reports in which e.g. the prevalence of *N. caninum* infected dairy cows in Sweden was 2% (Björkman *et al.*, 2000; Hemphill & Gottstein, 2000), and from Paper IV, also indicate that *N. caninum* is

less prevalent in Sweden than in many other European countries. There is no known explanation for this and several factors may have contributed to the current situation. One important circumstance is of course the low prevalence (0.5%) of *N. caninum* in Swedish dogs (Björkman, Lundén & Uggla, 1994). As the parasite is transmitted between the definitive and the intermediate hosts it is however impossible to determine if the low prevalence in dogs is a result of the low prevalence in cattle, or vice versa. Swedish cattle are typically housed indoors during the winter season and kept on pasture during summer (this is regulated by law). Because of the climate, fodder and straw are usually stored indoors. Usually, only dogs belonging to the farmer have access to storehouses and cattle accommodations. Dog faeces contaminating pasture or food and straw before harvest cannot be excluded. In this context it might be worth mentioning that stray dogs are not seen in Sweden.

It is not known whether there are definitive hosts of *N. caninum* in Sweden besides dogs. As there are reports about *N. caninum* infection in the red fox (Buxton *et al.*, 1997; Almeria *et al.*, 2002), and indications of the grey fox being a risk factor for *N. caninum* infection (Barling *et al.*, 2000), foxes could be suspected to be definitive hosts of the parasite. Although the red fox is spread all over Sweden no infected individuals were found in a survey of Swedish red foxes (Jakubek *et al.*, 2001). If future research shows that foxes excrete *N. caninum* oocysts, the apparent lack of infection in Swedish foxes would in part explain why horizontal spread is rare and constitute a favourable precondition for control of *N. caninum* infection in the country. In Swedish wildlife, another species that is more closely related to dogs and coyotes is the wolf (*Canis lupus*). However, although slightly increased during the last decade, the population of Swedish-Norwegian wolves is very small and present in only a few counties.

The Swedish isolate of *N. caninum* seems to be of low virulence in experimentally infected mice (Atkinson *et al.*, 1999). If this relatively low virulence is applicable also to bovine infections, this could be a reason why horizontal spread of *N. caninum* is not common in the country. However, these results are based on laboratory stocks from one single isolate and it remains to be shown that the parasites have the same characteristics in the field.

The optimal conditions for survival of *N. caninum* oocysts in the environment have not yet been described. There seems to be a slight tendency for lower prevalence of *N. caninum* in northern parts compared to southern parts of Europe (for a summary see Hemphill and Gottstein, 2000). In an epidemiological study from Germany, regions with higher summer temperatures had higher prevalence of *N. caninum* positive herds (Schares *et al.*, 2003). In Sweden, infected herds have been identified in the southern, middle and northern parts. The number of identified herds is however too small to make assessments about prevalence in different parts of the country. If high average temperature is a critical factor for the survival and sporulation conditions of *N. caninum* oocysts, the Swedish climate may impair the spread of *N. caninum*. Besides temperature and other climate parameters, isolation from the European mainland, a relatively low herd density and a low prevalence of other bovine infections are factors that could have contributed to the lower prevalence of *N. caninum* in Sweden. Also, the trading

pattern may be different from other countries with a higher prevalence of *N. caninum* infection.

## Control measures

The tests that were validated within this project constitute a useful set of diagnostic tools that can be readily applied in control schemes and in further research. From the results of this thesis and other research it seems that the prospects of controlling the presence of *N. caninum* infection in Sweden are favourable. With the right measures the risk of spread of the parasite within and between herds can be minimized.

Although prevalence is low, infected individuals constitute a potential risk for spread of the parasite due to vertical transmission. Further, interaction with a definitive host can result in a new outbreak of horizontal transmission. Measures aimed at eliminating infection in a herd should be combined with preventive measures to avoid re-infection. Infection status should be given a high priority in the culling decision process. In cases of low within-herd prevalence immediate culling of infected cows should be considered. Furthermore, the within-herd prevalence can be consistently reduced by making sure that test-positive heifers are not kept for replacement. In case new animals are purchased, these should first be tested and declared free from *N. caninum* infection.

It is desirable that horizontal transmission is reduced by control of definitive hosts. Although dogs are confirmed to shed *N. caninum* oocysts, we have no clear proof of the source of postnatal infection in the Swedish herds investigated. Dogs are still the main suspects and it is important that dogs are treated as a risk factor for *N. caninum* infection. In other words, they should not be allowed to contaminate fodder or straw. Placentas, aborted foetuses, raw meat or slaughter offal from infected cows should not be given to dogs or wild carnivores.

# Implications for future research on *N. caninum* infection

Since *N. caninum* in cattle was first described and named, the Swedish research group has contributed to and taken an active part in international research collaboration. Because this parasite is relatively recently discovered there is still a considerable need for further studies within this field of research.

### *National screening of bulk milk samples*

To estimate the herd prevalence of *N. caninum* in Sweden, screening of a large number of bulk milk samples from all parts of the country should be performed. From these results it would be possible to study how infected herds are distributed within this country. This kind of investigation will be particularly interesting since Sweden is a country with a considerable difference in climate between southern and northern parts. It is also desirable that the results be compared to geographical data, prevalence of dogs and various species of wildlife.

Estimates of *N. caninum* prevalence in non-Swedish populations, based on the bulk milk iscom ELISA, would make it possible to compare results in relation to different epidemiological preconditions. Results from foreign populations, perhaps with higher prevalence of infection, might also be utilized to learn more about the performance (especially Se) of this test.

### *Investigation of the Swedish population of beef cattle*

*N. caninum* infection causes economic losses in both dairy and beef cattle. In a beef herd, an abortion outbreak could seriously damage the production of a whole season. Once introduced, the parasite can remain in a herd for several years and thereby increase the risk of abortions. When a calf is aborted a major part of the production of a beef cow is lost. There are no estimates available about the prevalence of *N. caninum* in the Swedish population of beef cattle. In order to retrieve this information, serological screening is needed. If it is decided to apply control measures on dairy cattle, it follows that the beef cattle population should eventually be included.

### *Studies of infection dynamics*

In order to determine when and where horizontal transmission occurs, more frequent repeated samples are needed from herds at risk. A study that follows calves from birth and during their first years would be preferable. The design of such a study should also make it possible to clarify how within-herd animal movements affect the risk of transmission.

### *Possible association with other bovine infections*

It is difficult to explain why peaks of abortion problems occur in herds with endemic *N. caninum* infection. It has been suggested that disorders in the immune system could activate latent *N. caninum* infection. In a Swedish investigation of dairy cows that had aborted, there was a significant association between presence of antibodies to *N. caninum* and BVDV (Björkman *et al.*, 2000). Therefore, it would be logical to also investigate the possible association with other bovine infections that may cause a temporary decrease in immunocompetence or cause abortion in cattle. Infectious agents of interest could be other viruses, e.g. bovine corona virus, bovine respiratory syncytial virus and bovine herpes virus type 1, or agents like *Chlamydophila* spp.

### *Consequences of* N. caninum *infection*

Although there are a few estimates of the economic impact of *N. caninum* infection in other countries, it would be of great value for Swedish farmers to have estimates directly applicable to their situation. There is also a general need to further investigate the consequences of *N. caninum* infection. Studies should include investigations of effects on fertility and reproduction, as well as milk production. International reports about the effect of *N. caninum* infection on milk production have been contradictory (reviewed in Dubey, 2003).

### *Development of a* N. caninum *vaccine*

As *N. caninum* infection seems to be persistent and as there is no treatment available, development of a vaccine would mean a major breakthrough. The prospects for this have been discussed by Innes *et al.* (2002). So far, one vaccine has been marketed in certain non-European countries (Barling *et al.*, 2003). However, protective immunity towards *N. caninum* has not been demonstrated with this vaccine. Under Swedish conditions, a vaccine would be best used in infected herds in order to prevent vertical transmission in infected cows. This way congenital infection could be prevented and valuable breeding material would not be wasted.

### *Additional* N. caninum *isolates from Swedish cattle or dogs*

New isolates of *N. caninum* are of great value for the international genetic epidemiology research. The fact that there is only one Swedish isolate, and that this is less virulent in mice and has a slower growth rate than other isolates, makes it particularly interesting to obtain additional isolates from Sweden.

### *Alternative definitive and intermediate hosts*

It is desirable to investigate potential hosts of *N. caninum* in Sweden. If available, serum or other body fluids, or faeces, might be used for indirect or direct demonstration of the parasite. Examples of interesting species are the wild ruminants; roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and European elk (*Alces alces*), as well as various carnivores and

omnivores; e.g. the wolf (*Canis lupus*), the bear (*Ursus arctos*), the badger (*Meles meles*), and the brown rat (*Rattus norvegicus*).

### *Avidity*

As mentioned in the background section of this thesis, measurement of antibody avidity is a relatively new diagnostic method in veterinary (and human) medicine. There is a need for further studies of its applicability and performance on both individual and herd level. An increasing interest also for avidity tests for other infections is foreseen, and development of such tests would be a valuable contribution to the diagnostic toolbox for many infections.

# Conclusions

The validation of the *N. caninum* iscom ELISA confirmed that this test has high Se and Sp when used to demonstrate antibodies in serum. By applying alternate cut-off levels, the test can be used for different applications. The modified version, that enables estimates of antibody avidity, can be utilized to create a herd avidity pattern that describes duration of infection. The iscom ELISA is also proved useful for bulk milk testing. Bulk milk test results are to some degree related to within-herd prevalence and can be used to categorize herds into groups with different probability of infection. Together, these three test versions constitute a valuable and flexible toolbox for future research and monitoring of *N. caninum* infection.

From the longitudinal study of infected Swedish dairy herds, it can be concluded that vertical transmission is the dominant route of transmission and that horizontal spread appears to be rare. Results from the regional screenings and previous research show that the prevalence of *N. caninum* in Sweden is low. However, each infected individual constitutes a potential risk for further spread of the parasite. The information gained from this thesis project indicates that the preconditions for reducing the prevalence of *N. caninum* in Swedish herds are favourable.

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# Acknowledgements

The studies were carried at out the Department of Ruminant Medicine and Veterinary Epidemiology, Faculty of Veterinary Medicine and Animal Science, SLU, Uppsala, Sweden. Financial support was provided by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, formerly SJFR), and the Swedish Farmers' Foundation for Agricultural Research (SLF). The studies were part of the EU research collaboration COST 820 and 854.

I would like to thank all who in any way contributed to this work over the years, and especially:

Rauni Niskanen, Head of the Department of Ruminant Medicine and Veterinary Epidemiology, for placing the facilities of the department at my disposal, and for your honest interest and support.

Camilla Björkman, my scientific supervisor, for sharing your genuine knowledge about *Neospora* and research in general. Thanks for helping me through it all, for resolving big or small problems, and for always keeping your door open for me to storm into your room with questions and ideas. You have been an excellent supervisor. Thanks for many good laughs and great teamwork!

Arvid Uggla, my associate supervisor, for taking your time, enduring my short dead-lines and giving me support when I needed it the most. Thanks also for your calm and positive approach and for making some of my more expensive project ideas possible. I have been very lucky to have you as my associate supervisor.

Brenda Bonnett, my associate supervisor, for encouragement and spot-on criticism of papers. I really wish you would have been present more often here in Uppsala but our e-mail correspondence has also been very valuable to me!

Ann Lindberg, my associate supervisor this last year, but very much involved in this project from the start. You had the ideas for some of the best parts of my work. Thanks for sharing your intelligence and positive energy! Thanks also for patiently explaining the information of the Swedish Dairy Association database and my epidemiology questions, over and over again.

Katarina Näslund, for careful analysis of thousands (!) of samples from my projects. Thanks for your valuable contribution to my work and for your patient and immediate help whenever I asked.

Eva-Britt Jakubek, for letting a novice like me into you lab. Thanks also for assisting and pep-talking me before my first big talk!

Jens Mattsson, for the best travelling company I could wish for and for leading our international collaboration with competence and a no-problem attitude. Last, but not least, for our discussions about life and mutual television friends ☺.

Susanne Stenlund, for good advice and "professional big sister" support. Thanks for sharing your data and, most of all, for being such a warm and good-natured person.

All my colleagues at the Department of Ruminant Medicine and Veterinary Epidemiology, for smiles and "hellos" in the corridors. Stefan Alenius, for sharing your solid experience of bulk milk testing and reading my papers, and Kicki Forslund for your concern about me and the rest of the PhD-students at our department.

The animal caretakers at our department, for excellent help during teaching and weekend work. Special thanks to Lotta, Kerstin, David and Bengt for assisting me at sampling of some herds, and Lotta and Kerstin also for handling hundreds of test result envelopes.

PhD-students at the Department of Ruminant Medicine and Veterinary Epidemiology. Ane, Ann, Ann-Charlotte, Aran, Charina, Jaruwan, Johanna, Karin, Marie and Sara – you're the best! Special thanks to Ann Arvidson, for sharing ideas about how to handle Stata and the Swedish Dairy Association database. Aran Chanlun, for discussions and shared interest

The farmers who participated in my project, for taking your time, allowing sampling of your cows and motivating me.

Veterinarians and AI technicians who sampled some of the herds in my project, especially Jan Sundberg, Elsa Blomgren, Katarina Gustafsson, Joakim Holmdahl, Torbjörn Kettisen, Rose-Marie Winroth and Andreas Sandin.

Lotta Söderström, for sending in gloves full of bulk milk samples, and Catarina Svensson for allowing me to contact the farmers in your project and to come to Skara to inform them about a certain parasite.

Per Arnesson and the staff at Skara Semin, for great collaboration. It's been a fascinating experience for me to start up such a big sampling machinery and end up at the desk with a big pile of valuable test results. You did all the work!

Sören Larsson, my mentor in "PUMA-projektet", for interesting discussions, for sharing your point of view and listening to mine.

Fellow *Neospora* researchers of the COST 820 and 854 collaborations. It has been a true inspiration to meet with you and to take part of your interesting work.

The Epidemiology discussion group, for discussions and brainstorming, for forcing me to read Rothman & Greenland… and for many nice chats!

Participants and teachers, i.e. Wayne Martin and Victoria Edge, at the NOVA courses in veterinary epidemiology in 1999 and 2000, for introducing me to epidemiology and making me feel that this is the most interesting field of research.

PhD-students of “Doktorandklubben”, for letting me join your group and taking care of me when I was the only PhD-student from IME around.

The talented women of "Smaklökarna", for eating, drinking, laughter, gossip and very serious discussions indeed.

Ulrika Grönlund for discussions about all aspects of life and for sharing Reference Manager output styles (=most important at this stage ☺).

Friends at Uppsala Budoklubb, for taking my mind off work, for making me feel good, and most importantly, for making me move!

Ulrika Wählby. Thanks for being a true friend and for keeping the "emergency line" open for me day and night. I miss you in Uppsala!

Åsa and Elin, for being outstanding sisters and inspiring role models. I'm so grateful to be a part of your team! With you by my side I know I will always be safe in life.

Mamma and Pappa, for supporting me no matter what and for continuing to look after me. Thanks for your generosity and common sense. I'm very proud to be your daughter.

Jimmy, for taking care of things and feeding me these past few months. Thanks also for loving me.