

Epidemiology of *Neospora caninum* Infection in Dairy Cattle in Thailand

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Abstract

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The protozoan parasite *Neospora caninum* is causing bovine abortion worldwide. The overall aim of this thesis was to gain a better understanding about *N. caninum* infection in dairy cattle in Thailand both at the individual animal and the herd level. Specifically, the aims were to investigate variations of *N. caninum* antibodies in milk of individual cows during lactation, to demonstrate the use of bulk milk antibody testing and its application, and to characterize *N. caninum* dynamics of infection in Thai dairy herds. The thesis is based on four separate studies.

The levels of *N. caninum* antibodies in milk of 15 infected cows varied considerably during 18 months although they were consistently considered positive. Cows of all lactation groups had a higher milk antibody at calving compared to at later months after calving, but the only significant difference was in the first lactation. Serum and milk antibody levels were always lower in first lactation than in second and later lactations. The results showed that individual milk can be an alternative material to demonstrate presence of *N. caninum* antibodies in lactating cows.

The bulk milk antibody levels in a cross-sectional sample of 11 herds ranged between 0.04 and 0.89 and the seroprevalences varied between 0% and 46%. Herds with higher bulk milk antibody levels showed a trend of higher portion of seropositive cows although there was no strong relationship between the bulk milk antibody level and the within-herd seroprevalence. Forty-six percent of the 220 bulk milk samples from nine milk collection centres were judged positive and the herd prevalence varied between milk collection centres.

Repeated bulk milk antibody testing of 418 dairy herds was evaluated and the herd *N. caninum* status was established at three consecutive samplings during one year. Herd status at either of the first two samplings was used to predict herd status at the last sampling, and was also interpreted in combinations. Using combinations gave higher predictability of a herd's *Neospora* status than a single test. One hundred and thirty-six were considered negative, and one hundred and thirty-four herds were positive throughout the study. It was concluded that repeated bulk milk testing at regular intervals provided better information about herd *N. caninum*-antibody status than a single test. The results also showed that the infection is prevalent in northeast Thailand, but that a herd can keep a negative infection status.

When 11 dairy herds were investigated repeatedly during four years, the overall percentage of antibody-positive cattle was constant and varied only between 10% and 13%. However, the within-herd seroprevalence differed substantially between herds. Vertical transmission, i.e. from dam to calf, was the most frequent route of transmission. The proportions of individual animals that changed from being seronegative to seropositive and from being seropositive to seronegative between the years were 3.9-4.6% and 19-39%, respectively, although most animals had consistent serological status throughout the study. Some herds can thus keep free from *N. caninum* infection without farmers taking control measures.

Key words: *Neospora caninum*, Iscom ELISA, Prevalence, Bulk milk, Herd status, Dairy cattle, Thailand, GEE, Milk, Serum, Cattle.

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Appendix

Papers I-IV

This thesis is based on the following four papers:

- I. Chanlun, A., Emanuelson, U., Aiumlamai, S., Björkman, C. 2006. Variations of *Neospora caninum* antibody levels in milk during lactation in dairy cows. *Veterinary Parasitology* (In press).
- II. Chanlun, A., Näslund, K., Aiumlamai, S., Björkman, C. 2002. Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand. *Veterinary Parasitology* 110, 35-44.
- III. Chanlun, A., Emanuelson, U., Chanlun, S., Aiumlamai, S., Björkman, C. 2006. Application of repeated bulk milk testing for identification of infection dynamics of *Neospora caninum* in Thai dairy herds. *Veterinary Parasitology* 136, 243-250.
- IV. Chanlun, A., Emanuelson, U., Frössling, J., Aiumlamai, S., Björkman, C. 2006. A longitudinal study of change in seroprevalence and seroconversion of *Neospora caninum* infection in dairy cattle in northeast Thailand. Submitted for publication.

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Abbreviations

ANOVA	analysis of variance
BVDV	bovine viral diarrhoea virus
DPO	Dairy Farming Promotion Organisation of Thailand
ELISA	enzyme-linked immunosorbent assay
E.T.	embryo transfer
GEE	generalised estimation equations
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
IgG	immunoglobulin G
IHC	immunohistochemistry
iscom	immunostimulating complex
<i>N. caninum</i>	<i>Neospora caninum</i>
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNV	predictive negative value
PP	percent positivity
PPV	predictive positive value
Se	sensitivity
Sp	specificity

Background

The parasite

The intracellular protozoan *Neospora caninum* is a cyst-forming parasite, belonging to the family Sarcocystidae within the phylum Apicomplexa. It was first identified in dogs with encephalomyelitis and myositis in 1984 (Bjerkås, Mohn & Presthus, 1984) and was named in 1988 (Dubey *et al.*, 1988). However, later retrospective studies have revealed *N. caninum* parasites in dogs as early as 1957 (Dubey, Koestner & Piper, 1990) and in a stillborn calf in 1974 (Dubey, Hartley & Lindsay, 1990). *N. caninum* is closely related to *Toxoplasma gondii*, but the two parasites are distinct in both ultrastructure and antigenicity (Dubey & Lindsay, 1989; Lindsay *et al.*, 1993; Speer *et al.*, 1999). *N. caninum* from dogs and aborted bovine foetuses are shown to be the same species (Jardine, 1996; Marsh *et al.*, 1995; Stenlund *et al.*, 1997).

Biology and life cycle

Three infective stages of *N. caninum*, i.e. tachyzoites, bradyzoites and oocysts, have been identified (Lindsay, Dubey & Duncan, 1999; McAllister *et al.*, 1998). Tachyzoites and bradyzoites are asexual stages of the parasite and are found intracellularly. The tachyzoites are lunate, ovoid, or globular in shape, 3 to 7 x 1 to 5 µm in size. They can be found in different cell types and organs, most often in the brain and spinal cord (Dubey & Lindsay, 1996). In pregnant cattle, tachyzoites have been found in the placenta (Piergili Fioretti *et al.*, 2000; Shivaprasad, Ely & Dubey, 1989). The tachyzoites can multiply rapidly in the host cell resulting in cell death and necrotic lesions (Dubey & Lindsay, 1996). Bradyzoites are 7 x 2 µm in size and are primarily found in the central nervous system and other neural tissues. They usually inhabit tissue cysts (Baszler *et al.*, 1996; Dubey *et al.*, 1990). Tissue cysts are round to oval in shape, up to 107 µm in diameter and with a cyst wall up to 4 µm thick. Oocysts are the result of sexual reproduction of *N. caninum* and are spherical to subspherical and 10-11 µm in diameter. Non-infective unsporulated oocysts are shed in faeces of a definitive host and sporulate and become infective within three days in the environment (Lindsay, Dubey & Duncan, 1999; Lindsay, Ritter & Brake, 2001; McAllister *et al.*, 1998). The sporulated oocysts have two sporocysts, each with four sporozoites. *N. caninum* oocysts are morphologically similar to *Hammondia heydorni* found in dog faeces, and *Toxoplasma gondii* and *Hammondia hammondi* found in cat faeces (Dubey, 1999b; Dubey *et al.*, 2002; Lindsay, Upton & Dubey, 1999).

N. caninum has a two-host life cycle, including intermediate and definitive hosts. Dogs and coyotes are the definitive host for the parasite (Gondim *et al.*, 2004c; McAllister *et al.*, 1998). Wolves have also been suggested as a definitive host but it has not been verified that they excrete *N. caninum* oocysts (Gondim *et al.*, 2004b). Dogs become infected and shed oocysts with their faeces after ingesting infected tissues of intermediate hosts (Basso *et al.*, 2001; Dijkstra *et al.*, 2001; Gondim *et al.*, 2004c; Lindsay, Dubey & Duncan, 1999; McAllister *et al.*,

1998). Recently, it was shown that a puppy can produce and shed more oocysts than an adult dog (Gondim, McAllister & Gao, 2005). Cattle and other animal species such as water buffaloes, pigs, red foxes, white-tailed deer, gerbils and monkeys can serve as intermediate hosts of the parasite (Almeria *et al.*, 2002; Dubey, 1999b; Gondim, 2006; Guarino *et al.*, 2000; Vianna *et al.*, 2005). The intermediate hosts become infected after intake of oocysts-contaminated feed and water, or by eating tissues of infected animals (Gondim *et al.*, 2004a; McAllister *et al.*, 2000; McAllister *et al.*, 1996). There are still uncertainties concerning the survival of oocysts in the environment.

Neosporosis in dogs

The most severe cases of canine neosporosis have been seen in young, congenitally infected puppies, especially during their first six months of life (Dubey & Lindsay, 1996; Peters, Wagner & Schares, 2000).

Characteristically, they show a progressive paresis or a severe paralysis of the hind limbs caused by severe polymyositis, polyradiculitis and disseminated meningoencephalitis. Furthermore, an ascending paralysis is a common clinical sign in both puppies and bitches (Dubey, 2003). Cutaneous neosporosis manifested by pyogranulomatous ulcerative dermatitis has also been reported (Perl *et al.*, 1998). Congenital infection in dogs has been demonstrated but is less common than post-natal infection and parasites can infect the foetuses in subsequent pregnancies (Barber & Trees, 1998; Dubey & Lindsay, 1990).

Neosporosis in cattle

Prevalence

N. caninum infection in cattle has been reported from several countries over the world. Neosporosis is considered as the most frequently diagnosed cause of abortion in cattle worldwide (Dubey, 2005). It seems that the prevalence of *N. caninum* in cattle differs between countries and regions (Bartels *et al.*, 2006a). Moreover, seroprevalence estimates of *N. caninum* infection vary considerably between studies. In Europe, between 16% and 83% of dairy herds were confirmed to have *N. caninum* infected animals (Bartels *et al.*, 2006a; Quintanilla-Gozalo *et al.*, 1999). However, the *N. caninum* seropositivity of cattle in the infected herds varies considerably and, in some dairy herds, up to 87% of cattle are seropositive (Frössling, Uggla & Björkman, 2005).

Symptoms

Abortion is the only clinical sign in adult cattle that are infected with *N. caninum*. Abortion can occur throughout pregnancy, but is most common between five to seven months of gestation (Anderson *et al.*, 1995; Wouda *et al.*, 1997). Abortion may take place throughout the year and may be epidemic, endemic, or sporadic (Anderson, Andrianarivo & Conrad, 2000). Epidemic abortion means that a large number of the pregnant cows in a herd experienced an abortion within a short

period of time, whereas the term endemic abortion refers to the situation when abortions occur throughout the year, and during several years. Experimental studies have shown the importance of the timing of *N. caninum* infection during pregnancy. Thus infections in cattle during early gestation are more likely to cause foetal death and resorption rather than abortion (Barr *et al.*, 1994; Macaldowie *et al.*, 2004; Williams *et al.*, 2000). Infection at mid-gestation may result in abortion or the birth of a persistently infected calf (Guy *et al.*, 2001; Innes *et al.*, 2001; Maley *et al.*, 2003; Williams *et al.*, 2000). However, with infection in late pregnancy, cows usually deliver normally and most calves are clinically healthy but may be congenitally infected (Barr *et al.*, 1994; Guy *et al.*, 2001; Innes *et al.*, 2001; Williams *et al.*, 2000; Williams *et al.*, 2003). Seropositive cows do not necessarily abort although they may have a 3 to 19 times increased risk of abortion compared with their seronegative herd mates (Hall, Reichel & Ellis, 2005; López-Gatius, Pabon & Almería, 2004; Pfeiffer *et al.*, 2002; Thurmond & Hietala, 1997a; Wouda, Moen & Schukken, 1998). In one investigation it was also found that seropositive primiparous cows had the highest risk (i.e. 7.4 times higher) of abortion and this risk decreased in subsequent pregnancies (Thurmond & Hietala, 1997a). Repeated abortions can occur in the same cows, but its frequency is not known (Anderson *et al.*, 1995; Thurmond & Hietala, 1997a; Wouda, Moen & Schukken, 1998).

It has not yet been clarified whether *N. caninum* infection can result in reproductive problems in the earliest stages of pregnancy. Some studies have found that seropositive heifers or cows required more inseminations per confirmed pregnancy than their seronegative herd mates (Gondim, McAllister & Gao, 2005; Hall, Reichel & Ellis, 2005; Munoz-Zanzi, Thurmond & Hietala, 2004). This may indicate that early foetal loss has occurred. However, a Spanish study suggests that *N. caninum* infection in animals chronically infected before pregnancy does not seem to affect the foetus survival during early pregnancy (López-Gatius, Pabon & Almería, 2004).

Most calves born to infected heifers or cows are clinically normal, but persistently infected with the parasite (Paré, Thurmond & Hietala, 1996). However, congenitally infected calves carried to term may also be stillborn, or alive but underweight, unable to rise and exhibit variable neurological deficits. These neurological signs consist of ataxia, with decreased patella reflex and a slight loss of conscious proprioception in either hind limbs, or all limbs (Dubey, 1999b). It has also been reported that some congenitally infected calves with good health at birth become progressively weaker within a few weeks after birth. Additionally, exophthalmia or an asymmetrical appearance of eyes has been observed (Dubey, 1999b).

Epidemiology

Epidemiological data suggest that cattle can become *N. caninum*-infected by both vertical and horizontal transmission. Vertical transmission, i.e. transplacental, is the most frequent route of *N. caninum* infection in cattle (Björkman *et al.*, 1996; Davison, Otter & Trees, 1999a; Frössling, Ugglå & Björkman, 2005; Schares *et*

al., 1998). Little is known of the mechanism for this. Repeated vertical transmission can occur in subsequent pregnancies and a congenitally infected heifer can herself give birth to congenitally infected calves (Björkman *et al.*, 1996; Frössling, Uggla & Björkman, 2005; Schares *et al.*, 1998; Wouda, Moen & Schukken, 1998). By congenital transmission, *N. caninum* infection can keep spreading in a herd for several years.

Mathematical modelling studies have indicated that vertical transmission alone cannot sustain *N. caninum* infection within a cattle herd. Horizontal transmission, i.e. that animals become infected after birth, is essentially required to maintain infection within a herd (French *et al.*, 1999). Horizontal acquisition has experimentally been verified to occur showing that cattle may be infected when they consume oocysts (Gondim *et al.*, 2004a) shed by dogs or coyotes (Gondim *et al.*, 2004c; McAllister *et al.*, 1998). Dogs become infected and shed the oocysts in faeces within two weeks after consuming tissues (Gondim, Gao & McAllister, 2002), or placenta (Dijkstra *et al.*, 2001) from cattle infected with *N. caninum*. Experimental studies have shown that tachyzoites in colostrum or milk replacer can orally infect newborn calves (Davison *et al.*, 2001; Uggla *et al.*, 1998). However, the parasites have never been found in colostrum or milk from naturally infected cows. Cattle can probably be infected by ingesting tissues that contain tachyzoites or tissue cysts (Piergili Fioretti *et al.*, 2000; Shivaprasad, Ely & Dubey, 1989).

Diagnosis

The most definitive evidence of *N. caninum* infection is isolation of the viable parasite by cultivation in cell culture and inoculated mice. However, many attempts to viable isolate were unsuccessful because most *N. caninum* in fetuses die with the host (Dubey, 2005).

Histopathological examinations of bovine foetal tissues combined with immunohistochemistry (IHC) are frequently used methods for demonstration of *N. caninum* infection. The presence of the parasite in the tissue is a confirmation of infection but the sparse distribution of parasites makes detection difficult. Brain, liver, and heart are essentially required for histology although lesions of neosporosis can also be found in other organs (Collantes-Fernandez *et al.*, 2006; Dubey, 1999b; Dubey & Lindsay, 1996; Wouda *et al.*, 1997). IHC methods utilizing specific antibodies to *N. caninum* are used to identify the parasites. This method is highly specific, but is laborious and has a low sensitivity (Gonzalez *et al.*, 1999; Gottstein *et al.*, 1998; Otter *et al.*, 1995).

Several methods of polymerase chain reaction (PCR) have been developed to amplify *N. caninum* DNA in formalin-fixed, paraffin-embedded, or fresh tissues from aborted bovine fetuses (Baszler *et al.*, 1999; Ellis *et al.*, 1999; Pitel *et al.*, 2001; Sager *et al.*, 2001). These techniques have also been used to detect *N. caninum* infection in different bovine tissues such as brain, spinal cord, heart (Davison *et al.*, 2001; Sager *et al.*, 2001), and placentas (Bergeron *et al.*, 2001; Ho *et al.*, 1997; Innes *et al.*, 2001; Piergili Fioretti *et al.*, 2000), as well as in amniotic

fluid of infected cattle (Ho *et al.*, 1996; Ho *et al.*, 1997). Additionally, PCR has been applied to detect *N. caninum* oocysts in dog faeces (Basso *et al.*, 2001; Gondim *et al.*, 2004a; Hill *et al.*, 2001). PCR assays are highly sensitive and specific, and less affected by autolysis than are histopathology.

Presence of specific antibodies to *N. caninum* indicates that an individual is or has been infected with the parasite. A variety of antibody assays have been developed for demonstration of antibodies to *N. caninum*, including the indirect fluorescent antibody test (IFAT), immunoblot, direct agglutination test, and enzyme-linked immunosorbent assays (ELISA). For a review, see Björkman & Uggla (1999).

Antibody assays can be used to diagnose *N. caninum* infection in aborted foetuses. For this purpose, it can be applicable on foetal blood and body fluids e.g. plural and peritoneal fluids. However, different studies report a low sensitivity when foetal fluid has been analysed by either IFAT or ELISA (Gottstein *et al.*, 1998; Reichel & Drake, 1996; Slotved, Jensen & Lind, 1999; Wouda, Dubey & Jenkins, 1997). Both the age and the stage of the autolysis of foetus can affect the sensitivity of the assays. False negative results can be seen in young foetuses because the immune system is not fully developed before the bovine foetus is six months old (Barr *et al.*, 1995; Gonzalez *et al.*, 1999; Wouda, Dubey & Jenkins, 1997). Moreover, severe autolysis can cause degradation of the immunoglobulins of the foetuses (Wouda, Dubey & Jenkins, 1997). It has been suggested that the use of immunoblot can improve the sensitivity and specificity of foetal *N. caninum* serology (Söndgen *et al.*, 2001).

Serology might be applied on newborn calves to determine whether they are congenitally infected. Because colostral antibodies from the dam may persist in the calf for several months (Hietala & Thurmond, 1999; Wouda, Moen & Schukken, 1998) it is important to collect a blood sample from the calf before consuming colostrum.

In adult animals, serum is the most commonly used material for demonstration of antibodies to *N. caninum*. However, *N. caninum* specific antibodies can also be found in vaginal secretion and saliva (Ooi *et al.*, 2000). In addition, milk can be utilized for antibody detection (Björkman, Holmdahl & Uggla, 1997; Schares *et al.*, 2004a). The antibody assay can also be applied when determining specific antibodies to the parasite in bulk milk from dairy herds (Bartels *et al.*, 2005; Björkman *et al.*, 2000; Frössling, Lindberg & Björkman, 2006; Schares *et al.*, 2004b; Schares *et al.*, 2003; Varcasia *et al.*, 2006).

Moreover, serology is an invaluable tool to determine whether an aborting cow or a herd experiencing an abortion problem has been exposed to *N. caninum* (Scharas *et al.*, 2002). However, careful consideration should always be given when interpreting the serologic results (Anderson, Andrianarivo & Conrad, 2000; Jenkins *et al.*, 2002). It is generally accepted that most naturally infected cows are seropositive at the time of abortion (Paré, Thurmond & Hietala, 1997; Stenlund *et al.*, 1999). However, as the antibody levels fluctuate during pregnancy (Stenlund

et al., 1999), an infected cow may, sometimes, be seronegative (Conrad *et al.*, 1993; Cox, Reichel & Griffiths, 1998; Dannatt, 1997; Wouda *et al.*, 1998).

Antibody assays, i.e. IgG avidity ELISAs, have also been developed to discriminate between chronic and acute infections caused by *N. caninum* (Aguado-Martínez *et al.*, 2005; Björkman *et al.*, 1999; Maley *et al.*, 2001; Sager *et al.*, 2003; Schares *et al.*, 2002). The principle of the IgG avidity test is that the binding strength of *N. caninum* antibodies (affinity) increases in chronically infected animals. A low avidity value is thus indicative of a recent infection. The avidity iscom ELISA has been previously applied in naturally *N. caninum* infected cattle herds to estimate the duration of infection and to elucidate the infection pattern in the herds (Björkman *et al.*, 2003; Dijkstra *et al.*, 2002a; Jenkins *et al.*, 2000; McAllister *et al.*, 2000). It has also been used to assess the duration of infection in cattle inoculated orally with *N. caninum* oocysts (Björkman *et al.*, 2005).

Economic impact

Neosporosis is recognized to be a cause of substantial economic losses to both the dairy and the beef industry, but there are no conclusive data on its economic impact. Abortion appears to be the main cause of economic loss and some estimations of direct and indirect costs of *Neospora*-associated abortions have been reported (Chi *et al.*, 2002; Dubey, 1999a; Trees *et al.*, 1999). In North America, it has been estimated that the economic loss due to *Neospora*-associated abortions in California is \$35 million per year (Dubey, 1999a) and in the Maritime provinces of Canada \$1064 annually (Chi *et al.*, 2002). An average annual loss as a consequence of *Neospora*-associated abortion outbreaks in New Zealand has been estimated to approximately \$15.7 million at a national and \$4451 at an individual farm level (Antony & Williamson, 2001). In Europe, direct economic losses due to *N. caninum* infection have been estimated to be \$11 million per year in Swiss dairy cattle (Häsler *et al.*, 2006) and \$2572 annually in Dutch dairy herds that experienced *N. caninum*-associated abortion epidemic (Bartels *et al.*, 2006b). Moreover, replacement cost for the infected herds can increase because heifers that experienced an abortion are more likely to be culled than their non-aborting herd mates (Paré, Thurmond & Hietala, 1997; Pfeiffer *et al.*, 2002; Thurmond & Hietala, 1996; Thurmond & Hietala, 1997a).

N. caninum infection may also affect milk production in dairy cows. In two North American studies it was found that seropositive cows produced 345-363 kg less milk per 305-day lactation than their seronegative herdmates (Hernandez, Risco & Donovan, 2001; Thurmond & Hietala, 1997b), representing a loss of approximately \$128/seropositive lactating cow (Hernandez, Risco & Donovan, 2001). However, Keefe & VanLeeuwen (2000) reported from an investigation on 90 Canadian herds that seropositive cows produced slightly more milk than seronegative cows. Other effects of neosporosis that can cause economic losses are, e.g. repeat breeding, loss of foetuses, stillbirth or birth of weak calves (Trees *et al.*, 1999; Waldner *et al.*, 2001; Waldner, Janzen & Ribble, 1998).

Prevention and control

A major route of *N. caninum* infection in cattle herds is from a dam to her foetus. A basic approach to reduce the infection rate is therefore to remove infected cows. The rationale for culling is that most infected cows can be expected to give birth to an infected calf, and that congenital infection appears to be life-long (Björkman *et al.*, 1996; Frössling, Ugglå & Björkman, 2005; Paré, Thurmond & Hietala, 1996). Recently it was shown that test-and-culling was a successful strategy to control *N. caninum* infection in Australian dairy herds (Hall, Reichel & Ellis, 2005).

Not breeding replacement heifers from infected cows is another strategy to control *N. caninum* infection in herds with a high rate of vertical transmission. This reduces the number of infected animals in the herd by blocking transplacental infection. In addition, both purchase and selective retention of seronegative heifer calves can reduce the prevalence of seropositive animals in the herds (Davison, Otter & Trees, 1999a; French *et al.*, 1999; Hall, Reichel & Ellis, 2005; Hietala & Thurmond, 1999). Compared with culling of infected cows, this approach is often a less costly alternative. Experimental data have indicated that *N. caninum* is not transmitted by embryo transfer (E.T.) from seropositive donors to seronegative cows (Campero *et al.*, 2003). Thus, E.T can be an alternative method to prevent congenital infection if only seronegative recipients are used (Baillargeon *et al.*, 2001; Landmann *et al.*, 2002).

It is also important to minimize the risk of infection by dogs. Specific measures would include removal of aborted fetuses, dead calves, and placentas from infected cows so that dogs cannot eat them. Exposure of cows to faeces of definitive hosts could be reduced by minimizing the number of dogs (and other suspected intermediate hosts, e.g. other canids) in the herd, covering feeds and commodities to prevent contamination by dog faeces (Baillargeon *et al.*, 2001; Dijkstra *et al.*, 2002b; McAllister *et al.*, 2000; McAllister *et al.*, 2005).

There is no drug or chemotherapy available for treatment of bovine *N. caninum* infection or to prevent transmission of the parasite from an infected dam to her offspring. A killed *N. caninum* vaccine is commercially available in the USA. However, it has not yet been shown that this vaccine is efficient in preventing vertical infection or abortion (Andrianarivo *et al.*, 2000; Barling *et al.*, 2003; Romero, Perez & Frankena, 2004).

Dairy farming in Thailand

Dairy farming in Thailand was first established in early 1960s in the Saraburi and Rachaburi provinces in the central region. The Thai Danish Farm Training Centre was established at Muak lek in Saraburi, as a joint venture project between Thai and Danish Governments. In 1971, it was reorganized into a state-owned enterprise named “The Dairy Farming Promotion Organisation of Thailand” (DPO). Since the beginning of the 1990s, the Thai government strongly has encouraged the smallholder dairy farms also in other parts of the country,

including the northeast and the north regions. Because more cows were needed to meet the increasing demand for milk and dairy products, pregnant cattle were imported from other countries, especially from Australia and New Zealand. In parallel, crossbred cattle were produced within the country. Because milk production of the crossbred cows is still low, frozen semen of proven pure breed sires are now imported from other countries to improve the productivity of their offspring.

Usually, dairy farmers join and form a dairy cooperative at either provincial or district levels to operate and manage milk collection and business. Most cooperatives sell their milk to either DPO, or private companies that possess processing plants. Moreover, many cooperatives process their own milk and operate for selling to the market themselves. Ready-to-drink milk consumption increased during the last decade and the Thai Government in 1998 launched a school milk programme which provides free milk for all elementary school students. Demands of milk products in the country always exceed the supply therefore dairy products, particularly milk powder, are imported.

The most serious problems that often occur on dairy farms are low fertility and low conception rate, high incidence of mastitis and low milk yield. There is usually shortage of high quality roughage (e.g. silage or hay) to feed the animal, especially during the dry season. In the rainy season roughage is usually enough to feed the animals. Most farmers allow their cattle to graze during the rainy season although fresh grass is usually cut, carried and given to the cattle.

In 2005, the total number of dairy cattle in Thailand was 478,836 heads (<http://www.dld.go.th/ict/yearly/yearly48/book/stat/stat02.xls>; 01-Sep-2006). The average herd size in smallholder farms is approximately 10-15 lactating cows.

Bulk milk antibody testing

Bulk milk antibody testing has been used for detection of infection with a variety of agents such as viruses (Emanuelson, Anderson & Alenius, 1989; Lindberg *et al.*, 2001; Niskanen *et al.*, 1991), bacteria (Nielsen *et al.*, 2000; Veling *et al.*, 2001) and nematode parasites (Kloosterman *et al.*, 1993; Sanchez & Dohoo, 2002). It has also been applied on control programmes for bovine diarrhoea virus (BVDV) in e.g. Scandinavia, and a part of Austria (Alenius, Lindberg & Larsson, 1997; Hult & Lindberg, 2005; Lindberg & Alenius, 1999; Rikula *et al.*, 2005; Rossmannith, Janacek & Wilhelm, 2005; Valle *et al.*, 2005). For different viral diseases, studies have shown a good correlation between levels of antibodies in bulk milk and prevalence of antibody-positive cows (Elvander *et al.*, 1995; Niskanen *et al.*, 1989; Pritchard, Kirkwood & Sayers, 2002). Moreover, some of the studies revealed that the stage of lactation did not substantially affect the test performance (Niskanen *et al.*, 1989; Pritchard, Kirkwood & Sayers, 2002). Recently, bulk milk antibody testing was evaluated to be used to identify the *N. caninum* infected herds (Bartels *et al.*, 2005; Frössling, Lindberg & Björkman, 2006).

Aims

The overall aim of this thesis was to gain a better understanding about *Neospora caninum* infection in dairy cattle in Thailand both at the individual animal and the herd level.

The specific objectives were:

- To determine the variation in *N. caninum* antibody level in individual milk during lactation in dairy cows and investigate the association between antibody levels in serum and milk of the same cows (**I**).
- To evaluate the application of repeated bulk milk antibody testing to establish the *N. caninum* antibody status of dairy herds (**II, III**).
- To investigate the prevalence of *N. caninum* infection in different dairy farming areas (**II, III**).
- To investigate infection dynamics and changes in prevalence of *N. caninum* in smallholder dairy herds in Thailand, and to use serological data to investigate how the within-herd seroprevalence changes over time and demonstrate seroconversions in individual cattle (**III, IV**).

Materials and methods

Detailed description of different study designs, materials and methods used are given in each paper (I-IV).

Animals and herds

The dairy herds included in this thesis were all owned by smallholder farmers in northeast or north Thailand. They were located nearby or within a village and/or close to other farms. Most cattle were of Holstein-Friesian crossbreed and were housed in open buildings. The newborn calves were separated from their dams immediately after birth and usually given the first colostrum milked from their dams within a few hours. The calves were then kept in restricted pens until weaning at about 2 months of age when they were transferred to the area for young stock. Heifers and cows were kept together and fed roughage and concentrate and were usually allowed to graze even though fresh grass was cut, carried and given to them during the rainy season. Rice straw was given as roughage during the dry season when there is usually a shortage of green grass.

In **Paper I**, 15 antibody-positive cows selected from three herds were used in a prospective study between February 2003 and September 2004 to investigate the variation in *N. caninum* antibody level in milk during lactation, and to study the association between antibody level in serum and milk. One of the herds (Herd 7) also participated in studies presented in **Paper II** and **IV**. Each farm was visited every 2-3 months and blood and milk samples were collected at the same time.

In **Paper II**, 11 dairy herds were used in a cross-sectional investigation to determine the relationship between the antibody level in bulk milk and the seroprevalence of *N. caninum* infection in the milking cows. All cows that contributed to the bulk milk, 10-19 in each herd, were blood sampled once during August 2001. A longitudinal study was conducted in the same herds between August 2001 and November 2004 to further investigate the seroprevalence dynamics of *N. caninum* and to demonstrate patterns of seroconversion in individual cattle (**Paper IV**). Each herd was then visited once a year (in total 4 times). Most cattle above 3 months of age were blood sampled at each farm visit and at the same time samples were collected from the bulk milk. When farm dogs were present during the visit they were also sampled (n=36).

Paper II also comprised bulk milk samples from 220 dairy herds collected between May and August 2000 at seven milk collection centres in northeast and at two in north Thailand. The aim of this cross-sectional study was to assess the prevalence of *N. caninum* infection in dairy herds of milk collection centres within and between the two regions.

Further, in **Paper III**, a longitudinal study was performed in 418 dairy herds in the same seven milk collection centres in northeast Thailand to evaluate the

application of repeated bulk milk antibody tests to establish the *N. caninum*-antibody status of dairy herds. Bulk milk samples were collected at three consecutive samplings, in December 2002, April 2003, and December 2003.

Sample collection, treatment and storage

Blood samples from the coccygeal vein (**Paper I, II and IV**) and composite milk from individual cows (**Paper I**) were collected into sterile tubes. Bulk milk samples were collected either at the farms (**Paper II and IV**) or at milk collection centres (**Paper II and III**). Milk cans are usually delivered from the farms to the milk collection centres twice a day, after morning and afternoon milking. Bulk milk samples were collected after appropriate stirring in the milk can. Each farm could have more than one can (40 kg milk in each can) and then samples were obtained from all cans and subsequently pooled into one sample.

Blood samples were kept at the ambient temperature (approximately +30 °C) for 2-4 h whereas milk samples were immediately put into an ice box (+4 to +8 °C). All samples were delivered to the laboratory during the same day as collection. All samples were centrifuged at 1000 x g for 15 min at +4 °C. Serum and skim milk were collected, inactivated at 56 °C for 30 min (**Paper I**) or 90 min (**Paper II-IV**) and stored at -20 °C until analysis.

Antibody detection

Presence of specific antibodies in bovine samples was analysed by *N. caninum* iscom ELISA as described by Björkman *et al.* (1997) and Frössling *et al.* (2003) (**Paper II-IV**). In **Paper I**, a commercial version of the iscom ELISA (SVANOVIR[®] Neospora-Ab iscom ELISA, Svanova Biotech AB, Uppsala, Sweden) was used. Sera and milk were diluted 1:100 and 1:2, respectively, in PBS-Tween before analysis. Positive and negative control sera were heat-treated in the same manner as the samples and included in each analysis as controls. All samples and controls were analysed in duplicates and the optical density (OD) was measured at 450 nm. Mean OD values of the duplicates were calculated and subsequently correlated to a positive control serum. The results were presented as corrected absorbance values (**Paper II-IV**) or percent positivity (PP) (**Paper I**).

The dog sera was analysed according to Björkman *et al.* (1994) (**Paper IV**).

Models and statistical tests

Multilevel models for evaluation variation in antibody level in individual milk and serum of antibody-positive cows (Paper I)

Multilevel models were used to assess the variation of antibody levels in milk and serum of the individual cows. The individual measurements were considered as the level-1 units and the individual cows were the level-2 units. The models were constructed using the generalized estimating equations analysis. The sampled

herd, time between calving and sampling, lactation number, season and serum PP were included as explanatory variables. The interaction between lactation number and month after calving was also evaluated in all models. An adjusted mean of the outcome variables was calculated for all combinations of lactation number and month after calving based on the estimated parameters to illustrate the variation during lactation as well as the variation in serum PP. The adjustments were done to the average level of all other explanatory variables.

*Comparison of differences in *N. caninum* infection between milk collection centres and between regions (Paper II)*

Differences in mean bulk milk absorbance between the milk collection centres and the regions were analysed by analysis of variance (ANOVA). The two-sample test for proportions was used to compare the number of bulk milk samples in different classes between regions.

Evaluation of repeated bulk milk antibody testing (Paper III)

The bulk milk OD were dichotomized at 3 different cut-off levels in order to establish herd *N. caninum*-antibody status as negative or positive. The results at first two samplings were used to predict the herd status at the last sampling. The predictive abilities were determined using methods commonly applied in evaluation of diagnostic tests, i.e. the sensitivity (Se), specificity (Sp), predictive negative (PNV) and positive (PPV) value (Thrusfield, 1995). The test results of the first samplings were also interpreted in combination to either increase the PNV (termed “parallel” interpretation) or PPV (termed “serial” interpretation) (Thrusfield, 1995). A combined test result was created based on the test results at the first two samplings. In Combination A, a herd was considered negative if the status at both samplings were considered negative and positive otherwise, i.e. parallel interpretation. In Combination B, a herd was considered positive if a herd status at both samplings were considered positive and negative otherwise, i.e. serial interpretation.

Investigation of cow-offspring pairs (Paper IV)

The association between the serological status in dams and their offspring was measured and compared using 2x2 tables and Fisher’s exact test. To study the mode of *N. caninum* transmissions during the years each dam-offspring pair was allowed to contribute to the comparison only once, i.e. only the test results from the first sampling where both the dam and the offspring were present were considered.

Apparent vertical transmission rates were calculated as the proportion of antibody-positive dams that had antibody-positive offspring and the horizontal transmission rates as the proportion of antibody-positive offspring that had antibody-negative dams. The seropositive and seronegative seroconversion rates were calculated as described by Pan *et al.* (2004).

Results and discussion

Variation in milk antibody levels during lactation

There was a large variation of antibody levels both in milk and serum during lactation in the 15 antibody-positive cows (**Paper I**). However, the milk PP of all cows was always above the cut-off (PP=20) for the test.

Lactation number and month after calving had a significant effect on antibody levels in all models ($P < 0.001$). Cows of all lactation groups had a higher adjusted mean of milk PP at the month of calving (3-10 days after parturition) compared to later months after calving although the only significant difference was in first lactation. This is in agreement with a previous report by Schares *et al.* (2004a) showing that stage of lactation affects the *N. caninum* antibody levels in individual milk. Several studies have shown a considerable change in the total immunoglobulin (Ig) levels in lacteal secretions of cows around calving (Butler, 1994; Caffin, Poutrel & Rainard, 1983; Guidry *et al.*, 1980; Madsen *et al.*, 2004). Accumulation of Igs occurs during the last few weeks of pregnancy, and then the Ig level declines rapidly during the first two weeks after parturition. Thus a decrease in milk PP as lactation proceeds can be expected. The significant decrease in milk PP some months after calving found here can also be a result of the dilution effect of the increased milk production (Kloosterman *et al.*, 1993; Sanchez *et al.*, 2004). The increase in milk PP in later stages of lactation can be due to a decrease in milk yield together with the increase in milk immunoglobulin G (IgG) concentration when increasing time between calving and sampling (Caffin, Poutrel & Rainard, 1983; Guidry *et al.*, 1980). It has been suggested that the increase of oestrogen during gestation may cause release of *N. caninum* from the cells of infected cows, resulting in an enhanced *N. caninum* antibody production (Stenlund *et al.*, 1999). However, the increased milk PP observed here is probably not attributable to reproductive hormones since no significant fluctuation of serum PP across lactation was observed.

One of the cows was seronegative but her milk PP was consistently positive throughout the study. It is difficult to explain which factors affect *N. caninum* antibody levels in milk and serum of this cow. There are some contradictory findings showing that the udder health of cows might influence the concentration of IgG in milk. A significant increase of total IgG1 concentrations was found in quarters infected with *Staphylococcus aureus* (Caffin, Poutrel & Rainard, 1983). It has also been reported that milk from cows with subclinical mastitis can occasionally produce false positive reactions in milk ELISA for detection of antibodies to bovine leukaemia virus (Klintevall *et al.*, 1991). However, Niskanen *et al.* (1989) found no influence of somatic cell content in milk on BVDV-ELISA test results when they analysed milk from udder quarters with considerable different cell counts. All milk samples in the present investigation (**Paper I**) were apparently normal and we did not test any cow for subclinical mastitis. It is not known whether subclinical mastitis (i.e. high cell counts) affects *N. caninum*

ELISA tests. Further, we have no information regarding the agreement in *N. caninum* antibody levels between the four udder quarters of the same cow.

The adjusted mean of milk PP of cows classed in PP_SERUM ≥ 55 was significantly higher than that of cows classed in lower serum PP groups. The association between the serum and milk PP supports previous results by e.g. Ooi *et al.* (2000). The finding also confirms previous reports that individual milk samples of lactating cows can be used as alternative materials for either screening or diagnosis of *N. caninum* infection (Schaes *et al.*, 2004a; Varcasia *et al.*, 2006). The use of milk samples is not only simpler and non-invasive for animals than blood sampling, but also more cost-effective since the farmers themselves can collect the milk sample and deliver it to the laboratory. Because there are still uncertainties about whether subclinical mastitis affects the test result, composite milk is recommended for detection of *N. caninum* antibodies at individual lactating cows.

Evaluation of bulk milk antibody testing

When samples of the bulk milk together with sera from all lactating cows in 11 Thai dairy herds were analysed by *N. caninum* iscom ELISA, there was a large variation of the bulk milk absorbances (0.04-0.89) and the seroprevalences in the herds varied between 0% and 46% (**Paper II**). Herds with higher bulk milk absorbance showed a trend of higher percentage of seropositive cows. For example, a low bulk milk absorbance was found in the two herds in which all cows were seronegative to *N. caninum*. Further, the two herds that had the highest bulk milk absorbances (0.85 and 0.89) both had a high seroprevalence, i.e. 30% and 46%, among the lactating cows. There was a large variation of the bulk milk OD among the four herds that had only one single seropositive cow. Bulk milk is a pooled sample and represents all lactating cows. Many factors can therefore influence the performance of the test results e.g. the proportion of infected cows, milk yield and antibody levels of lactating cows (Frössling, Lindberg & Björkman, 2006) and lactation stage (Paper I; Schaes *et al.*, 2004a). It is worth noting that the studied herds were small, comprising only 10-19 lactating cows. Therefore, a single seropositive cow could considerably influence the bulk milk test result. The results indicated that bulk milk antibody testing can be used to identify *N. caninum*-infected herds. The results also suggested that a single bulk milk test result should be interpreted cautiously, especially in small herds where only a few cows contribute to the bulk milk and some seropositive cows may be dried-off.

In **Paper III** we investigated whether repeated bulk milk antibody testing could increase the predictability of a herd's true antibody status. When either sampling 1 or sampling 2 was used to predict herd status at sampling 3 (reference result), sampling 1 gave higher estimates of Se, but lower Sp than sampling 2 at all cut-offs. This is consistent with commonly observed patterns when evaluating performance of the diagnostic tests (Fletcher & Fletcher, 2005; Thrusfield, 1995) because it is well recognized that Se and Sp are inversely related. The estimated Se and Sp were also affected by the sampling occasion which confirms the

importance of sampling interval. However, it was unexpected, and difficult to explain, that the Se at sampling 2 was lower than at sampling 1, since a higher Se and Sp would be expected at the sampling occasion closest to sampling 3.

The results showed that the performance of the test was improved when herd status at the first samplings was interpreted in combination (**Paper III**). Both Combination A and Combination B improved the estimated predictive values compared to using either the results of sampling 1 or sampling 2 alone. Combination A gave higher PNV but lower PPV than Combination B at all cut-offs. Further, the estimated PNV of both Combination A and Combination B were consistently higher when changing to a higher cut-off. The result indicated that Combination A can be used to confirm absence of *N. caninum* infection. On the other hand, the PPV of Combination B for all the 3 cut-offs was higher than the PPV of Combination A, but the estimated PPV of both Combination A and Combination B did not differ among the different cut-offs. Because Combination B improved the point estimate of PPV, it is therefore useful to confirm presence of infection. However, there is always a risk for misclassification of a herd's status because neither PPV nor PNV is perfect. Further, both PPV and PNV have some shortcomings in terms of evaluating test characteristics because they are considerably affected by the prevalence in the study population (Thrusfield, 1995).

When the iscom ELISA was previously evaluated for use on bulk milk, 0.20 was suggested as suitable cut-off to identify infected herds (Paper II; Frössling *et al.*, 2006). Using this cut-off, 134 herds were classified positive at all samplings, indicating that they had antibody-positive cows throughout the study. This is in agreement with international studies that have shown that *N. caninum* infection can be kept in cattle herds for a long period (Björkman *et al.*, 1996; Frössling, Uggla & Björkman, 2005; Schares *et al.*, 1998). Furthermore, 136 herds remained negative at all three samplings, suggesting that they had only non-infected cows, or a low proportion of antibody-positive cows.

About 35% of the herds studied changed their bulk milk antibody status during the study. Specifically, 14% of the 158 herds considered negative at the first two samplings were positive at sampling 3. This change in antibody status could be caused by a chronically infected heifer or cow that did not contribute to the bulk milk at the first samplings but that contributed at the third sampling, or an antibody-positive cow that had been purchased between the samplings. On the other hand, 16% of the herds considered positive at sampling 1 and sampling 2 were negative at sampling 3, indicating that most or all of the antibody-positive cows were removed from the milking group. They might have been either in a dry period, or have been culled from the herd, but no detailed information about culling was available.

The bulk milk is a pooled sample of all lactating cows which contributed to the sample proportionally to their milk yield and antibody concentration. The use of single bulk milk testing result can lead to misclassification of the herd antibody-status because of the bulk milk result excludes information about non-lactating cattle (e.g. dry cows, heifers and calves) and lactating cows that do not contribute

to the bulk milk. However, bulk milk antibody testing at regular intervals provides better information about herd *N. caninum* infection status than a single test. Moreover, bulk milk antibody testing is a suitable tool for farmers to monitor their herd status over time.

***N. caninum* infection in Thai dairy cattle**

Prevalence of N. caninum infection in different regions

In total, 102 (46%) and 52 (24%) of 220 bulk milk samples collected from northeast and north Thailand in 2000 had absorbance values ≥ 0.20 and ≥ 0.50 , respectively (**Paper II**). Using a 0.20 cut-off to establish herd *N. caninum* antibody status, the prevalence of infected herds in the northeast and the northern regions were 43% and 52%, respectively. There was no difference in herd prevalence between the two regions but the north had a higher proportion of herds with bulk milk absorbance values ≥ 0.50 . Moreover, the proportion of infected herds varied considerably, i.e. between 25% and 55%, between the nine milk collection centres.

When repeated bulk milk testing of 418 dairy herds was later performed in the northeast the overall proportion of positive herds was 44-57% (**Paper III**). In addition, the prevalence of the infected herds decreased slightly from sampling 1 to sampling 3 in all but one of the milk collection centres. Notably, no information about sampling results was given to the farmers during the study. A possible explanation for this decrease could be the structural change in the dairy herds in the areas implying that old and possibly problem-cows (i.e. high risk for *N. caninum* positive) were most likely to be removed.

Together these findings suggest that a high proportion of Thai dairy herds harbour *N. caninum*-infected cows and that the infection is widespread in both north and northeast Thailand. Cattle in the north have a higher risk of being infected compared to those in the northeast but the reason for this difference between the regions is not known.

Dynamics of N. caninum infection

The results of the present study showed a constant overall percentage of *N. caninum* antibody-positive cattle over four years, varying between 10% and 13%, although the within-herd prevalence differed between the herds (**Paper IV**). Previous investigations in Thailand have shown that the prevalence of *N. caninum* infection differs both between studies and between regions. It has been reported that about 6% of the dairy cattle in the central region of Thailand have antibodies to *N. caninum* (Kyaw *et al.*, 2004; Suteeraparp *et al.*, 1999), whereas a higher seroprevalence, 12 to 70%, has been found in the northeast (Kashiwazaki *et al.*, 2001; Suteeraparp *et al.*, 1999). Furthermore, a rapid increase in seroprevalence found in the latter area was suggested to be a result of postnatal infection (Kashiwazaki *et al.*, 2001). In Thailand *Neospora*-tachyzoites have been identified in the placenta of a seropositive aborting cow by IHC examination (Kyaw *et al.*,

2003). Later, a tissue cyst was found in the brain of a calf born to a seronegative cow and the DNA sequence of the parasite confirmed to be identical with the reference strain NC1 (Kyaw *et al.*, 2005). However, it is not known whether the parasite present in different parts of the country is similar to isolates that have been characterized in other countries.

Of the 424 individuals that were sampled more than once during the four years, 344 (81%) and 32 (8%) were consistently test-negative and test-positive, respectively (**Paper IV**). However, both seropositive and seronegative conversions in individual animals were observed and interpretation of these results is complicated. Seropositive conversions in cattle due to postnatal infection, recrudescence of a chronic *N. caninum* infection or false-positive test results have been reported from other countries (see e.g. Dijkstra *et al.*, 2002a; Dijkstra *et al.*, 2003; Frössling, Uggla & Björkman, 2005; Gondim *et al.*, 2004c; Hietala & Thurmond, 1999; Pfeiffer *et al.*, 2002; Waldner *et al.*, 2001). The proportions of animals that in the present investigation changed from being seronegative to seropositive between the years (3.9-4.6%) was consistent with two Canadian studies (Pan *et al.*, 2004; Waldner *et al.*, 2001). Even higher rates of seropositive conversion (45-55%) have been reported from the Netherlands (Dijkstra *et al.*, 2002a) and Australia (Pfeiffer *et al.*, 2002). Based on samples from Swedish *N. caninum*-infected herds, the specificity of the iscom ELISA has been estimated to be high but not perfect (Frössling *et al.*, 2003). Some test-positive animals in the present study could thus be expected to be false-positives. However, the number of inexplicable positive results was higher than expected. One explanation for this could be that nonspecific cross-reactivity, e.g. to other infectious agents present in Thailand but not in northern Europe, might have altered the performance of the test and given a somewhat higher portion of false positive results.

In **Paper IV**, the high proportions of animals which changed from being seropositive to seronegative between the years (19-39%) were consistent with some other researchers' findings (Pan *et al.*, 2004; Sager *et al.*, 2001; Waldner *et al.*, 2001). In a Canadian study, 65% of 81 seropositive cows were seronegative when they were sampled again after 2 years (Pan *et al.*, 2004). Although most of the conversions in that study took place in animals that were moderately positive at the first sampling, also strongly seropositive individuals became seronegative. In addition, a study in Thailand has recently reported that 100% (4/4) of *N. caninum*-infected cows were seronegative at calving about 1 year after they had tested seropositive (Kyaw *et al.*, 2005). However, Dijkstra *et al.* (2003) found that only 4% of 616 seropositive cattle in 21 *Neospora*-infected herds seroconverted over a 2-year period, and suggested that most of the conversions were due to false positive or negative test results, or that the individual initially tested positive because of remaining maternal antibodies. Moreover, transient false positive results of 2- to 3-year-old heifers classified as antibody-negative have been reported (Hietala & Thurmond, 1999). *N. caninum* antibody levels can fluctuate during pregnancy (Stenlund *et al.*, 1999) and may also fall below the cut off of the test (Dannatt, 1997). Recently, a study has revealed an increase of *N. caninum* antibody titres in cows after they consumed oocysts, which later reverted to seronegative status (Gondim *et al.*, 2004a). It has generally been considered that

N. caninum infection is life-long. However, it is difficult to explain how seronegative conversion can occur between subsequent samplings to such extent as in this and in other studies, unless *N. caninum*-infected animal can become free of the parasite after infection. Together with previous findings our results suggest that some cattle can indeed get rid of the infection.

Transmission of N. caninum in Thai dairy herds

Vertical transmission was the most common route of *N. caninum* infection in the two herds with consistently high within-herd prevalence (**Paper IV**). Vertical transmission over several generations was also observed in herds with moderate seroprevalence, corroborating that *N. caninum* can remain in cattle herds for generations by this transmission route (Björkman *et al.*, 1996; Frössling, Uggla & Björkman, 2005). The apparent vertical transmission rate of 58% in these 11 herds was similar to the 56% found in a Spanish investigation (Mainar-Jaime *et al.*, 1999). However, congenital transmission rates over 80% (Davison, Otter & Trees, 1999a; Hietala & Thurmond, 1999; Paré, Thurmond & Hietala, 1996) and transmission rates below 45% have also been reported (Bergeron *et al.*, 2000; Pan, *et al.*, 2004). The apparent rates of vertical transmission could be either underestimates or overestimates because of the sampling schedule. The serological status of either the dam or its offspring, or both, may have changed since the time of birth of the offspring. Such problems can be avoided by testing offspring immediately after birth but before consuming colostrum in order to minimize possible classification. It is worth noting that each dam-offspring pair was allowed to contribute to the comparison only once, i.e. only the test results from the first sampling where both the dam and the offspring were present were considered.

Horizontal transmission was also recorded although its source could not be identified and the infection rate was low (**Paper IV**). Notably only 4 of the 36 tested farm dogs had antibodies to *N. caninum* and all seropositive dogs were present in herds with a low seroprevalence. Nonetheless, it cannot be excluded that dogs were the possible source of horizontal infections. Dogs and coyotes are the definitive host of the parasite (Gondim *et al.*, 2004c; McAllister *et al.*, 1998) but coyotes are apparently absent in Thailand. Dogs can become infected and shed oocysts after ingestion of infected tissues (Gondim, Gao & McAllister, 2002) or placenta (Dijkstra *et al.*, 2001) from *N. caninum* infected cattle. Studies have shown that antibody-negative dogs can also excrete oocysts and be a risk factor for infection (Dijkstra *et al.*, 2001; Lindsay, Ritter & Brake, 2001; McAllister *et al.*, 1998). In addition, rats have been presumed to be able to serve as a reservoir for *N. caninum* infection on the cattle farm after finding DNA of the parasites in captured brown rats (*Rattus norvegicus*) (Huang *et al.*, 2004; Hughes *et al.*, 2006). Other species such as poultries, ducks, cats, rodents and birds are always present on the cattle herds in Thailand. It is not known whether any of these species plays any important role in spread of *N. caninum* infection in Thai population of dairy cattle.

Several studies have shown that *N. caninum*-seropositive cows have higher risk of abortion than seronegative herdmates (Davison, Otter & Trees, 1999b; Paré,

Thurmond & Hietala, 1997; Thurmond & Hietala, 1997a; Wouda, Moen & Schukken, 1998). In the present investigation we did not aim at investigating consequences of *N. caninum* infection. Two antibody-positive cows aborted and were culled shortly after experiencing the abortion (**Paper I**). Unfortunately, we were not able to retrieve any samples from the aborted foetuses for definitive diagnosis of *N. caninum* infection. However, most antibody-positive cows did not abort (**Paper I**, and **IV**), corroborating that cattle can harbour a chronic infection without showing any clinical signs (Björkman *et al.*, 1996). Moreover, three antibody-positive cows had also been culled due to reproductive problems, supporting the hypothesis that the infected cows could be culled due to reproductive problems without farmers knowing that they are *N. caninum* antibody positive (Thurmond & Hietala, 1996). Probably *N. caninum* causes early embryonic death and early foetal abortion resulting in an increasing number of inseminations (Gondim, McAllister & Gao, 2005; Hall, Reichel & Ellis, 2005; Munoz-Zanzi, Thurmond & Hietala, 2004).

The farmers in this study were not informed about which of their cows were seropositive nor given any information about how to control the infection. In **Paper IV**, no seropositive animals were found in 2 of the 11 herds (Herds 4 and 9) at the last two samplings, after the antibody-positive cattle were culled between sampling two and three. This finding supports the notion that culling of infected cattle can rapidly reduce the prevalence of infection, especially in herds with a low rate of postnatal transmission (French *et al.*, 1999; Hall, Reichel & Ellis, 2005; Romero & Frankena, 2003).

Suggestions for future research

Investigation of seroconversions

Results from the present investigation revealed that both seronegative and seropositive conversions occur in Thai dairy cattle. Seropositive conversion has been reported and discussed in several studies worldwide, but only little information is available about seronegative conversions. A long-term study with shorter intervals between blood samplings than we used here, would make it possible to more precisely determine when cattle convert their serological status. Such a study should be designed to make it possible to verify whether both congenitally and postnatally infected individuals can become free of the infection. Factors influencing the occurrence of such situation should be identified e.g. strain of the parasite, possible source of infection, or immune status of cattle.

*Demonstration of *N. caninum* isolates from Thailand*

The present investigation showed that *N. caninum* infection is widespread in different dairy farming areas of Thailand. Demonstration of the parasites and their DNA from both cattle and dogs should be valuable to confirm infection. Isolation of viable parasites is not always successful. However, PCR is now recognized as a specific sensitive technique used to identify species-specific *N. caninum* DNA sequences and it can be used to examine presence of *N. caninum* DNA in a variety of samples. PCR is preferable to isolation of viable parasites in terms of costs, speed and preparation of samples (i.e. type and volume). Moreover, PCR should be a suitable method to examine genotype and genetic diversity of *N. caninum* isolates especially from clinical cases. However, isolation of viable parasites is also necessary to elucidate their biological diversity. Different isolates of *N. caninum* have now been obtained from countries throughout the world and biological biodiversity among isolates has been proved in both *in vivo* and *in vitro* studies. Cattle infected by a low virulent isolate may not abort. In addition, it would be useful to determine whether the parasites present in Thailand are related to isolates in other countries e.g. Australia and New Zealand from where pregnant cattle were introduced into dairy farms of Thailand.

*Investigation of *N. caninum* infection in the Thai population of beef cattle*

N. caninum infection is well recognized as an important cause of abortion causing substantial economic losses in both dairy and beef cattle. Seroprevalences varying from 9% to 26% has been reported in beef cattle and in some countries. Cattle in beef herds has been shown to be more likely to test *N. caninum* seropositive than those in dairy herds. However, other studies have reported that seroprevalence of *N. caninum* in dairy cattle is higher than that in beef cattle. No information is available about the prevalence of the *N. caninum* infection in the Thai beef cattle population. The Royal Thai government has now started to promote rearing of beef cattle in many parts of the country, especially by smallholders, and more

investigations are therefore important to better understand epidemiology and control measures of the parasite in Thailand.

*Investigation of *N. caninum* infection in the Thai population of buffalo*

Serological evidence of *N. caninum* infection in water buffalo has been reported from some countries e.g. Egypt, Italy, Brazil and Vietnam. Moreover, the parasite was isolated from naturally infected water buffaloes in Brazil. In 2005 the total number of buffaloes in Thailand was 1,624,919 (http://www.dld.go.th/home/stat_L3.html; 01-Sep-2006); however, the prevalence of *N. caninum* infection in population of Thai buffalo is not known. Such an investigation is therefore of interest to determine whether the infection is prevalent in this species.

*Investigation of the relative importance of *N. caninum* in bovine abortion in Thailand*

Causes of abortion in cattle are complex and can be caused by both infectious and non-infectious agents e.g. virus, bacteria, fungi, toxic plants and parasites. *N. caninum* is well recognized to cause bovine abortions worldwide, but little is known about how it and other agents contribute to abortions in Thai cattle. It would be interesting to investigate the relative importance of *N. caninum* and other pathogens e.g. BVDV, bovine herpes virus type 1 and blood parasites on bovine abortions in Thailand. Veterinarians and extension staff should be informed and requested to submit samples from abortions e.g. bovine foetus and foetal membranes, blood and milk samples from the aborting cow to the laboratory for definitive diagnosis.

National screening of bulk milk samples

The present investigation showed the herd prevalence varied between dairy farming areas. This merits further investigations. Bulk milk samples from all parts of Thailand should be analysed to investigate the distribution of infected herds in the country.

Conclusions

- Milk can be used as an alternative material to demonstrate presence of *N. caninum* antibodies in lactating cows although the level of antibodies in milk of individual cows varied considerably during lactations.
- Bulk milk antibody testing is a useful tool to identify *N. caninum*-infected herds. Repeated bulk milk testing at regular intervals provide better information about *N. caninum*-antibody status of a herd than a single test, because a negative result of a single bulk milk test does not completely exclude the infection in herds.
- *N. caninum* appears to be prevalent in dairy herds in Thailand. Seroprevalence of *N. caninum* infection in Thai dairy cattle population was constant during the four years but variation of within-herd seroprevalence between herds was substantial.
- Vertical transmission seems to be the most frequent route of infection although postnatal infection may also contribute to the infection but is not common in most herds.
- Seronegative and seropositive conversions did occur in individual cattle although most animals had consistent serological status.
- A herd can keep a negative infection status despite the frequent presence of dogs. Herds with negative bulk milk antibody testing should be informed how they can keep free of infection. Newly purchased cattle should be serologically tested to avoid introducing an infected animal to the farms. Repeated bulk milk antibody testing should also be carried out every two to three years to monitor constancy of herd *N. caninum* status and infection dynamics.

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อรัญ จันทร์ลุน 2549 ระบาดวิทยาของการติดเชื้อ *Neospora caninum* ในโคนมในประเทศไทย วิทยานิพนธ์ระดับปริญญาคุณวุฒิปันจิติ

Neospora caninum เป็นเชื้อโปรโตซัวที่เจริญภายในเซลล์ พบการติดเชื้อนี้ในสัตว์หลายชนิด รวมทั้งโคและพบได้ในทุกภูมิภาคของโลก การติดเชื้อในโคเป็นสาเหตุของการแท้งที่สำคัญและส่งผลให้เกิดความสูญเสียทางเศรษฐกิจอย่างมาก การติดเชื้อจากแม่สู่ลูกผ่านทางรก (Vertical transmission) เป็นทางหลักของการติดเชื้อ *N. caninum* ในโค นอกจากนี้โคอาจติดเชื้อหลังเกิดได้ โดยการกินโอโอซิส (Oocysts) ของเชื้อที่ปนเปื้อนในน้ำหรืออาหาร (Horizontal transmission) โดยโอโอซิสเหล่านี้จะปนเปื้อนมากับอุจจาระของสุนัข การตรวจพบแอนติบอดีที่จำเพาะต่อเชื้อ *N. caninum* ในโคสามารถบ่งชี้ถึงการติดเชื้อได้ ดังนั้นวิธีการตรวจหาแอนติบอดีในซีรัมหรือน้ำนมจึงเป็นเครื่องมือที่สำคัญในการวินิจฉัยโรคและการศึกษาทางระบาดวิทยาของการติดเชื้อ *N. caninum* โดยถูกใช้เพื่อประเมินความชุก (Prevalence) ของการติดเชื้อ *N. caninum* ในโคนมในหลายประเทศรวมทั้งประเทศไทย วัตถุประสงค์ทั่วไปของวิทยานิพนธ์นี้คือ เพื่อศึกษาเกี่ยวกับการติดเชื้อ *N. caninum* ของโคนมในประเทศไทยทั้งระดับรายตัวและระดับฟาร์ม ส่วนวัตถุประสงค์เฉพาะได้แก่ เพื่อศึกษาการเปลี่ยนแปลงของระดับแอนติบอดีต่อเชื้อในน้ำนมของแม่โคในช่วงการให้นม เพื่อศึกษาการใช้วิธีการตรวจหาแอนติบอดีในน้ำนมถึงรวมฟาร์ม (Bulk milk antibody test) และการนำไปใช้ประโยชน์ และเพื่อศึกษารูปแบบและการเปลี่ยนแปลงการติดเชื้อของโคนมในฟาร์มในประเทศไทย ดังนั้นวิทยานิพนธ์นี้จึงประกอบด้วยรายงานการศึกษาวิจัยในหลายด้านที่จะทำให้ทราบและเข้าใจในสถานการณ์และรูปแบบของการติดเชื้อ *N. caninum* ในประเทศไทยได้ดียิ่งขึ้น โดยการตรวจหาแอนติบอดีต่อเชื้อ *N. caninum* ในตัวอย่างทั้งหมดด้วยเทคนิค iscom ELISA

ระดับแอนติบอดีต่อเชื้อ *N. caninum* ในน้ำนมโคทดลองรายตัว จำนวน 15 ตัว ให้ผลการทดสอบเป็นบวก ตลอดระยะเวลาศึกษา 18 เดือน แต่มีความแปรปรวนสูง โดยระดับแอนติบอดีในน้ำนมของโคที่อยู่ในระยะ 1 เดือนหลังคลอดสูงกว่าระยะการให้นมช่วงอื่นๆ และโคที่อยู่ในรอบการให้นมครั้งแรกเท่านั้นที่ระดับแอนติบอดีในระยะ 1 เดือนหลังคลอดแตกต่างจากระยะอื่นๆของการให้นม นอกจากนี้โคที่อยู่ในรอบการให้นมที่ 1 มีระดับแอนติบอดีในทั้งซีรัมและน้ำนมต่ำกว่าโคที่อยู่ในรอบการให้นมอื่นๆ ระดับแอนติบอดีในซีรัมและน้ำนมมีความสัมพันธ์กันอย่างมีนัยสำคัญ ($P < 0.001$) ดังนั้นตัวอย่างน้ำนมโครายตัวสามารถใช้เป็นทางเลือกเพื่อทดสอบหาแอนติบอดีต่อเชื้อ *N. caninum* ในแม่โคที่อยู่ในช่วงการให้นม

จากการศึกษาแบบตัดขวาง (Cross-sectional study) เพื่อหาความสัมพันธ์ระหว่างระดับแอนติบอดีต่อเชื้อ *N. caninum* ในน้ำนมถึงรวมฟาร์มและซีรัมของโครีดนมในฟาร์มโคนม จำนวน 11 แห่ง พบว่า ระดับแอนติบอดีในน้ำนมถึงรวมฟาร์มอยู่ในช่วง 0.40-0.89 เท่าของตัวอย่างควบคุมบวกและความชุก (Seroprevalence) ของโครีดนมที่ติดเชื้อในแต่ละฟาร์ม (เมื่อพิจารณาจากแอนติบอดีในซีรัม) อยู่ระหว่างร้อยละ 0 ถึง 46 โดยฟาร์มโคนมที่มีระดับแอนติบอดีในน้ำนมถึงรวมฟาร์มสูงกว่ามีแนวโน้มที่ความชุกของโครีดนมที่ติดเชื้อมากกว่า จากการตรวจแอนติบอดีในน้ำนมถึงรวมฟาร์มของฟาร์มโคนม จำนวน 220 แห่ง ที่เก็บจากศูนย์รวบรวมน้ำนมดิบ 7 แห่ง ในภาคตะวันออกเฉียงเหนือ และ 2 แห่ง ในภาคเหนือ พบว่าร้อยละ 46 ของฟาร์มโคนมทั้งหมดเป็นฟาร์มที่ติดเชื้อ (ฟาร์มที่มีระดับแอนติบอดีในน้ำนมถึงรวมฟาร์มมากกว่า 0.20 เท่าของตัวอย่างควบคุมบวก ถือว่าเป็นฟาร์มที่ติดเชื้อ) นอกจากนี้ความชุกของฟาร์มที่ติดเชื้อ (Herd prevalence) ของแต่ละศูนย์รวมน้ำนมดิบมีความแตกต่างกัน ดังนั้นการทดสอบหาแอนติบอดีต่อเชื้อ *N. caninum* ในน้ำนมถึงรวมฟาร์ม สามารถใช้เป็นเครื่องมือเพื่อค้นหาฟาร์มโคนมที่ติดเชื้อได้ นอกจากนี้มีฟาร์มโคนมจำนวนมากที่น่าจะมีโคนมที่ติดเชื้ออยู่ในฟาร์ม และการติดเชื้อมีการแพร่กระจายอยู่ทั่วไปในพื้นที่การเลี้ยงโคนมทั้งภาคเหนือและภาคตะวันออกเฉียงเหนือ

จากการศึกษาแบบไปข้างหน้า (Prospective study) ในฟาร์มโคนม จำนวน 418 แห่ง โดยเก็บตัวอย่างน้ำนมถึงรวมฟาร์ม จำนวน 3 ครั้ง ในระยะเวลา 1 ปี ห่างกันครั้งละ 4-6 เดือน เพื่อตรวจหาแอนติบอดีต่อเชื้อ *N. caninum* และใช้ผลการตรวจที่ได้ในแต่ละครั้งมากำหนดสถานภาพการติดเชื้อ *N. caninum* ของฟาร์ม โดยเทียบกับตัวอย่างควบคุมบวก ทั้งนี้เพื่อประเมินความเป็นไปได้ของการใช้ผลสถานภาพการติดเชื้อของฟาร์มจากการเก็บตัวอย่างครั้งที่ 1, หรือ 2 อย่างใดอย่างหนึ่ง และการใช้ผลสถานภาพการติดเชื้อของฟาร์มจากการเก็บตัวอย่างทั้งสองครั้งร่วมกัน เพื่อทำนายผลสถานภาพการติดเชื้อของฟาร์มในการเก็บตัวอย่างครั้งที่ 3 ผลการศึกษาพบว่า การใช้ผลสถานภาพการติดเชื้อของฟาร์มจากการตรวจน้ำนมถึงรวมฟาร์มครั้งที่ 1 และ 2 ร่วมกัน จะสามารถทำนายผลสถานภาพการติดเชื้อของฟาร์มในการเก็บตัวอย่างครั้งที่ 3 ได้ดีกว่าการใช้ผลสถานภาพของฟาร์มจากการตรวจครั้งที่ 1 หรือ ครั้งที่ 2 เพียงอย่างเดียวใดอย่างหนึ่ง ทั้งนี้หากใช้เกณฑ์ตัดสินฟาร์มที่ติดเชื้อตั้งที่กล่าวมาข้างต้นพบว่า จากการตรวจน้ำนมถึงรวมฟาร์มทั้งสามครั้งตลอดระยะเวลาการศึกษานี้ มีฟาร์มที่ไม่ติดเชื้อ 136 แห่ง และฟาร์มที่ติดเชื้อ 134 แห่ง ส่วนฟาร์มที่เหลือมีการเปลี่ยนแปลงสถานภาพการติดเชื้อของฟาร์มในการเก็บตัวอย่างอย่างน้อยหนึ่งครั้ง สรุปได้ว่าการตรวจหาแอนติบอดีต่อเชื้อ *N. caninum* ในน้ำนมถึงรวมฟาร์มอย่างต่อเนื่องสม่ำเสมอในระยะเวลาที่เหมาะสมจะทำให้มีข้อมูลที่สามารถทำนายสถานภาพการติดเชื้อของฟาร์มโคนมได้ดีกว่าการตรวจเพียงครั้งเดียว และผลการศึกษาแสดงให้เห็นว่าการติดเชื้อกระจายอยู่ทั่วไปและมีฟาร์มโคนมจำนวนมากในภาคตะวันออกเฉียงเหนือของประเทศไทยมีโคติดเชื้อ *N. caninum* อย่างไรก็ตามยังพบว่า มีฟาร์มโคนมจำนวนมากที่สามารถคงสถานภาพไม่ติดเชื้อในช่วงที่ดำเนินการศึกษานี้

เมื่อทำการศึกษาคัดเชื้อ *N. caninum* แบบยาวนาน (Long-term study) ในฟาร์มโคนม จำนวน 11 แห่งเป็นเวลา 4 ปี โดยการเข้าเยี่ยมฟาร์มปีละครั้ง และเก็บตัวอย่างซีรัมของโคนมที่มีอายุมากกว่า 3 เดือนขึ้นไป และสุนัขในฟาร์ม รวมทั้งน้ำนมถึงรวมฟาร์ม เพื่อตรวจหาแอนติบอดีต่อเชื้อ *N. caninum* ผลการศึกษาพบว่า ความชุกของโคที่ติดเชื้อของฟาร์มทั้งหมดค่อนข้างจะคงที่ อยู่ระหว่างร้อยละ 10 และ 13 อย่างไรก็ตามความชุกของโคที่ติดเชื้อในแต่ละฟาร์มแตกต่างกันมาก โดยค่าเฉลี่ยของความชุกของโคที่ติดเชื้อในแต่ละฟาร์มจากการตรวจ 4 ครั้งพบว่า ฟาร์มโคนม 2 แห่ง มีความชุกของโคที่ติดเชื้อมากกว่าร้อยละ 20 และระดับแอนติบอดีต่อเชื้อ *N. caninum* ในน้ำนมถึงรวมฟาร์มสูงทุกครั้งตลอดระยะเวลาการศึกษา และมีฟาร์ม 2 แห่ง ที่ไม่พบโคติดเชื้อ จากการตรวจซีรัมในการเก็บตัวอย่างสองครั้งล่าสุดและระดับแอนติบอดีในน้ำนมถึงรวมฟาร์มของฟาร์มทั้งสองอยู่ในระดับต่ำ เมื่อพิจารณาแนวโน้มของความชุกของโคที่ติดเชื้อในฟาร์มโคนมทั้ง 11 แห่ง พบว่า ความชุกของโคที่ติดเชื้อในฟาร์ม 5 แห่ง มีแนวโน้มลดลง ส่วนฟาร์มที่เหลือมีแนวโน้มที่ความชุกของโคที่ติดเชื้อจะมากขึ้นเมื่อสิ้นสุดการศึกษา อัตราการติดเชื้อ *N. caninum* จากแม่สู่ลูกที่พบในฟาร์ม คิดเป็นร้อยละ 58 ส่วนอัตราการติดเชื้อหลังเกิด คิดเป็นร้อยละ 5 ตลอดการศึกษานี้มีโคที่ถูกเก็บตัวอย่างซีรัมมากกว่า 1 ครั้ง จำนวน 424 ตัว โดยมีผลการตรวจแอนติบอดีทุกครั้งเป็นลบ 344 (81%) ตัว และเป็นบวก 32 (8%) ตัว ส่วนโคที่เหลือมีการเปลี่ยนแปลงสถานภาพการติดเชื้ออย่างน้อย 1 ครั้ง โดยพบโคที่เปลี่ยนสถานภาพการติดเชื้อจากที่เคยติดเชื้อเป็นไม่ติดเชื้อในแต่ละปี คิดเป็นร้อยละ 19 ถึง 39 ส่วนโคที่เปลี่ยนสถานภาพการติดเชื้อจากไม่ติดเชื้อเป็นติดเชื้อ คิดเป็นร้อยละ 3.9 ถึง 4.6 ต่อปี จากการตรวจซีรัมของสุนัขที่เลี้ยงอยู่ในฟาร์มโคนม พบว่า มีสุนัข 4 จากทั้งหมด 36 ตัวติดเชื้อ *N. caninum* โดยสุนัขที่ติดเชื้อทั้งหมดอยู่ในฟาร์มที่มีความชุกของโคที่ติดเชื้ออยู่ในระดับต่ำ ผลจากการศึกษานี้แสดงให้เห็นว่าโคส่วนใหญ่ไม่มีการเปลี่ยนแปลงสถานภาพการติดเชื้อ ดังนั้นฟาร์มโคนมบางแห่งอาจจะปลอดภัยจากการติดเชื้อ *N. caninum* ได้ โดยที่เกษตรกรไม่มีมาตรการควบคุมและป้องกันที่จำเพาะแต่อย่างใด



I





Short communication

Variations of *Neospora caninum* antibody levels in milk during lactation in dairy cows

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Abstract

A longitudinal study was performed to investigate the variations of *Neospora caninum* antibody levels in individual milk during lactation as well as the association between antibody levels in serum and milk. Serum and milk samples of 15 milking cows were collected between February 2003 and September 2004 in three smallholder dairy farms in Khon Kaen province in northeast Thailand. All samples were analyzed for presence of antibodies by an *N. caninum* iscom ELISA test kit and the results were given as percent positivity (PP). The effects of time between calving and sampling, lactation number, and season on milk and serum PP were studied using Generalized Estimation Equations methods. All cows were antibody positive in either milk or serum at the first two consecutive samplings. Although serum and milk PP varied considerably, milk PP was consistently positive throughout the study. Cows of all lactation groups had a higher adjusted mean of milk PP at calving compared to later months after calving although the only significant difference was in first lactation. Serum and milk PP were always lower in first lactation than in second and later lactations. An adjusted mean of milk PP for cows classified as having serum PP ≥ 55 was significantly ($P < 0.05$) higher than that of cows classified as having lower serum PP. Our results indicate that individual milk can be an alternative material to demonstrate presence of *N. caninum* antibodies in lactating cows.

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Keywords: GEE; Composite milk; Serum; Cattle; *Neospora caninum*; Iscom ELISA; Percent positivity

1. Introduction

Neospora caninum is an intracellular protozoan parasite well-recognized to cause endemic and epidemic bovine abortion throughout the world (Dubey,

1999). Transplacental transmission of the parasite is the most common route of infection. Up to 95% of *N. caninum* infected cattle transmit the infection to their offspring (Paré et al., 1997; Davison et al., 1999), and this can occur in consecutive pregnancies (Björkman et al., 1996; Frössling et al., 2005). Moreover, postnatal infection has been observed, probably caused by intake of feed and water contaminated with infective oocysts

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shed by the definitive hosts, i.e. dogs and coyotes (McAllister et al., 1998; Gondim et al., 2004), or infected tissues, e.g. placentae (Shivaprasad et al., 1989). Cows or heifers infected with *N. caninum* are two to seven times more likely to abort than uninfected animals (Dannatt, 1997; Paré et al., 1997; Moen et al., 1998). However, most infected fetuses are born clinically healthy but persistently infected. Such congenitally infected heifer calves can themselves transmit the parasite to their offspring (Björkman et al., 1996; Frössling et al., 2005).

Serological antibody test is the most common method used to diagnose whether animals have been exposed to the parasite (Björkman and Uggla, 1999; Jenkins et al., 2002) and some tests has been modified to enable analysis of milk (Björkman et al., 1997; Schares et al., 2004). Studies have shown that serum *N. caninum* antibody levels fluctuate in naturally infected cows during pregnancy with a general increase in mid-pregnancy and a decrease before calving (Paré et al., 1997; Stenlund et al., 1999; Williams et al., 2003). The antibody levels in infected animals may occasionally be below the cut-off for the test used (Dannatt, 1997).

Whereas changes in *N. caninum* antibodies in bovine serum are well documented, little is known about the variation of the antibody levels in milk of individual cows during lactation. This information is needed to clarify whether the *N. caninum* antibody levels in milk can be used as an alternative to antibody levels in serum when diagnosing *N. caninum* positivity. The objective of this study was to investigate the *N. caninum* antibody levels in individual milk during lactation in dairy cows. We further investigated the association between *N. caninum* antibody levels in serum and milk in the same cows.

2. Materials and methods

2.1. Study design

A longitudinal study was carried out between February 2003 and September 2004 in a convenience sample of three smallholder dairy farms connected with Muang milk collection centre in Khon Kaen province in northeast Thailand. Each farm was visited

every 2–3 months; in total 10 occasions during the study. The herd size varied between 10 and 20 milking cows, and between 4 and 6 cows were sampled in each herd. Cows eligible for inclusion in the study had to be clinically healthy and of Holstein–Friesian cross-breed. They were of various ages and at different stages of lactation. The cows selected were housed in the same open building and fed roughage and concentrate similar to their herd mates during the study. All were allowed to graze even though fresh grass was usually cut, carried and given to them during the rainy season. Rice straw was given as roughage during dry season since there is usually a shortage of green grass. At each visit, information about date of calving and lactation number were recorded.

2.2. Sample collection, treatment and storage

On each occasion, from each study cow, coccygeal vein blood and composite milk (15 ml) after mechanical milking were aseptically collected into a sterile tube. Blood samples were kept at the ambient temperature (+30 °C) for 2–4 h. Milk samples were immediately put into an ice box (+4 to +8 °C). Samples were delivered to the laboratory during the same day as collection. All samples were centrifuged at 1000 × g for 15 min at +4 °C. Serum and skim milk were collected, inactivated at 56 °C for 90 min and stored at –20 °C until analysis.

2.3. Antibody detection

The commercially available *N. caninum* iscom ELISA test kit (SVANOVIR[®] Svanova Biotech AB, Uppsala, Sweden) was used to demonstrate presence of immunoglobulin (Ig) G1 in serum and milk essentially following the manufacturer's instructions. The sera and milk were diluted 1:100 and 1:2, respectively, in PBS–Tween before analysis. The positive and negative sera provided with the test kit were heat-treated in the same manner as the samples and included in each analysis as controls. All samples and controls were analyzed in duplicates and the optical density (OD) was measured at 450 nm. The results were given as percent positivity (PP = [mean OD of sample/mean OD of positive control] × 100). Samples with PP ≥ 20 were considered positive (Varcasia et al., 2006).

2.4. Data analysis

A multilevel model was used to assess the variation of milk and serum PP at the individual cow level. Only cows considered as positive to *N. caninum* antibodies in either milk or serum at the first two consecutive samplings were included in the analysis ($n = 15$). We considered the individual measurements as the level-1 units and the individual cows as level-2 units, i.e. the identity of individual cows was included as a random effect. Three different models were constructed using the generalized estimating equations (GEE) analysis in Stata Statistical Software release 8.0 (StataCorp., College Station, TX, USA). In model 1, the milk PP (PP_MILK) was the outcome variable and the explanatory variables investigated were the sampled herd (HERD), time between calving and sampling (MONTH), lactation number (LACT) and season (SEASON). In model 2, all the explanatory variables included were identical to model 1 but, instead, serum PP (PP_SERUM) was the outcome variable. In model 3, both the outcome and explanatory variables included were identical to model 1 but the variable PP_SERUM was included as an explanatory variable to assess the potential effect of PP in serum on the variation of milk PP. The variable MONTH was categorized into five classes: 0 (month at calving), 1 (1–3 months after calving), 2 (4–6 months after calving), 3 (7–9 months after calving), 4 (10–12 months after calving) and 5 (≥ 13 months after calving); PARITY was three classes: 1 (lactation number = 1), 2 (lactation number = 2) and 3 (lactation number ≥ 3); SEASON was sampling dates categorized into four periods: January–March, April–June, July–September and October–December; PP_SERUM was categorized into three classes according to 33% percentiles: 0 (PP < 35), 1 (PP = 35–54) and 2 (PP ≥ 55). Due to the small number of herds that was selected, the variable HERD was used as a fixed-effect variable in the model. The interaction between LACT and MONTH was also evaluated in all three models. Repeated measurements were assumed to follow an exchangeable correlation structure within an individual. A robust estimate of the variance was used in the GEE model to minimize the possible effect of inaccurate estimates when the assumption of exchangeable correlation structure was violated (Dupont, 2002; Twisk, 2003). Measurements collected at >450 days in milk were excluded from the study to prevent using

information where date of calving was probably recorded incorrectly (irregularly) by the farmers. The value of 450 was calculated based on the average of previous calving intervals of the healthy cows with full term of pregnancy in the three herds studied.

Once each of the three models was fitted, an adjusted mean of the dependent variables was calculated for all combinations of LACT and MONTH based on the estimated parameters to illustrate the variation during lactation. Further, in model 3, an adjusted mean of the milk PP for PP_SERUM was also estimated to show the effect of serum PP on the variation of milk PP. The adjustments were done to the average level of all other explanatory variables.

3. Results

3.1. Descriptive statistics

Of the 15 cows studied, 10 remained throughout the study whereas 5 were culled due to reproductive problems (3) or sold to other herds (2). The range of the serum PP of the 15 cows was 9–111 with a mean of 57, whereas the milk PP varied from 21 to 119 with a mean of 74. More than 75% of serum and milk samples had PP ≥ 35 and PP ≥ 58 , respectively. All cows, except one, were seropositive throughout the study. Details of serum and milk PP according to all variables included in the GEE models are shown in Table 1. It was observed that one of the 15 cows sampled deviated considerably from the others. She had serum PP ranging from 9 to 21 whereas the milk PP was consistently positive over the study with a range of 24–71.

3.2. Multivariate analysis

The explanatory variable HERD was not statistically significant in any of the GEE models and SEASON was not significant in model 1 (Table 2). The variables were still retained in all models since they were regarded as biologically relevant and since non-significant variables do not influence the estimation of the effects of other variables. LACT and MONTH interacted significantly ($P < 0.001$) in all the three models. A histogram of the residuals from the models demonstrated that they were normally distributed,

Table 1
Descriptive statistics of *Neospora caninum* antibodies measured as percent of positivity (PP) in milk and serum samples of 15 cows of three dairy herds in northeast Thailand sampled between February 2003 and September 2004

Variables	Class	N ^a	PP in milk			PP in serum		
			Mean	S.D.	Range	Mean	S.D.	Range
Herd number	6	39	71	30	21–119	54	28	9–111
	7	24	74	19	38–112	63	21	18–97
	22	23	82	20	38–112	63	21	20–105
Month ^b after calving	0	7	98	16	69–119	63	31	31–111
	1–3	17	66	26	21–96	54	24	13–93
	4–6	16	62	29	24–99	50	24	12–80
	7–9	23	77	25	26–111	61	24	15–101
	10–12	17	78	20	43–112	55	25	9–87
	13–15	7	83	16	59–100	62	24	15–85
Lactation number	1	16	65	26	21–102	58	24	21–101
	2	20	88	13	58–119	69	17	35–111
	≥3	50	72	27	24–112	51	26	9–92
Season of test	January–March	21	72	22	21–102	57	25	9–101
	April–June	25	76	24	27–104	58	23	15–87
	July–September	25	75	30	26–119	53	28	12–111
	October–December	15	75	23	24–111	60	21	19–93
PP in serum class	<35	18	46	23	21–98	21	7	9–32
	35–54	16	58	17	27–95	40	4	35–47
	≥55	52	89	14	54–119	74	11	56–111

Presence of antibodies was demonstrated by an *N. caninum* iscom ELISA test kit.

^a N: number of observation.

^b The actual days between calving and sampling were 3–10, 32–89, 93–176, 185–270, 277–346 and 377–430 in the six classes of month, respectively.

Table 2
Fixed-effect Chi-square and *P*-value for percent positivity (PP) of *Neospora caninum* antibodies from three Generalized Estimation Equation models using PP in milk and PP in serum as dependent variables, respectively (cows = 15)

Variables	DF ^a	Model 1 ^b		Model 2 ^c		Model 3 ^b	
		Chi-square	<i>P</i>	Chi-square	<i>P</i>	Chi-square	<i>P</i>
Month after calving	5	633.90	0.000	21.65	0.001	292.68	0.000
Lactation number	2	59.43	0.000	8.86	0.012	24.71	0.000
Season of test	3	4.56	0.207	17.20	0.001	11.23	0.011
Herd number	2	0.44	0.804	0.30	0.860	1.80	0.407
PP in serum class	2	– ^d	–	–	–	32.97	0.000
LACT × MO ^e	10	865.97	0.000	77.23	0.000	727.71	0.000

Presence of antibodies was demonstrated by an *N. caninum* iscom ELISA test kit.

^a Degree of freedom.

^b PP in milk was used as dependent variable.

^c PP in serum was used as dependent variable.

^d Variable not included in the model.

^e LACT × MO indicates the effect of interaction between lactation number and month after calving.

showing that the linear model analysis of PP values was appropriate. In addition, plotting residuals against normal scores at the individual level had a good fit, i.e. yielding a diagonal straight-line plot.

An adjusted mean of the dependent variables of all the three GEE models across lactations are shown in Fig. 1. In model 1, the adjusted mean of milk PP at month of calving for cows in first lactation was significantly ($P < 0.05$) higher than that of cows at 1–3 months after calving and thereafter, but no differences in the adjusted means of milk PP of cows between 1 and 15 months after calving were observed (Fig. 1a). Cows in second lactation had a significant ($P < 0.05$) increase in the adjusted mean of milk PP at 10–12 months compared with at 7–9 months after calving. In addition, cows in their third and greater lactations had a significantly ($P < 0.05$) higher

adjusted mean of milk PP at month of calving than cows at 4–6 months after calving. Moreover, the adjusted milk PP of cows at 13–15 months after calving was significantly ($P < 0.05$) higher than that of cows at 1–6 months after calving.

In model 2, serum PP of cows within lactation numbers did not differ significantly throughout the study (Fig. 1b).

Changes in the adjusted mean of milk PP of the 15 cows as estimated in model 3, when including PP_SERUM as an explanatory variable, are shown in Fig. 1c. The patterns of changes of cows across all lactations were similar to model 1. The only difference being that the adjusted means of cows in third and later lactations at 13–15 months of lactation also was significantly ($P < 0.05$) higher than that of cows at 7–9 months after calving.

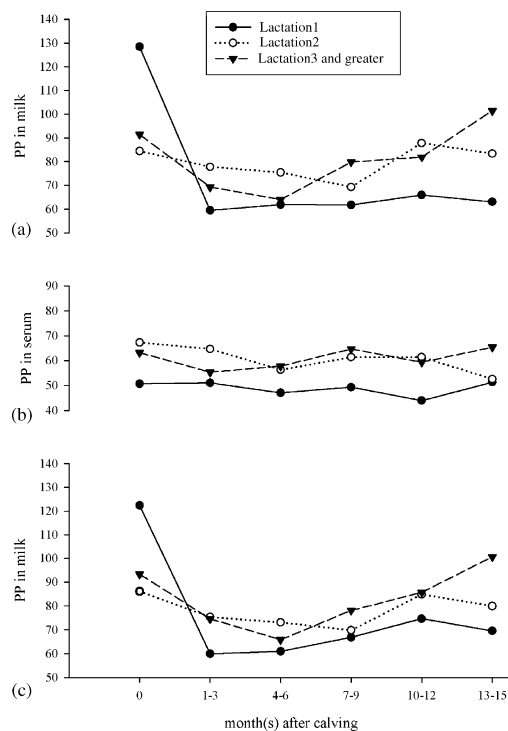


Fig. 1. Adjusted means of percent positivity (PP) of *Neospora caninum* antibodies in milk (a), serum (b), and milk adjusted also for differences in antibodies in serum (c) as estimated in a Generalized Estimation Equation model analysis of 15 milking cows. Presence of antibodies was analyzed by an *N. caninum* iscom ELISA test kit.

The adjusted mean of PP_MILK between cows classed in PP_SERUM <35 (mean = 50, CI: 40, 60) and 35–54 (61, CI: 81, 94) did not differ, but both were significantly ($P < 0.05$) lower than the mean of cows classed in PP_SERUM ≥ 55 (87, CI: 81, 94).

4. Discussion

The present study showed serum PP varied considerably, especially milk PP. However, the milk PP was always above the cut-off used for all cows. Moreover, all except one cow had serum PP that was considered positive throughout the study. The GEE models showed significant changes in milk PP within lactations since MONTH (time between calving date and sampling) was identified as an important factor. This is in accordance with the results of Schares et al. (2004) showing that stage of lactation affected the levels of *N. caninum* antibodies in milk samples. Cows of all lactation groups had a higher adjusted mean of milk PP at calving compared to later months after calving although the only significant difference was in first lactation. The cows sampled at months of calving, i.e. MONTH = 0, were sampled 3–10 days after parturition. Studies have shown a considerable change in the total Ig levels in lacteal secretions of cows around calving (Guidry et al., 1980; Caffin et al., 1983; Butler, 1994; Madsen et al., 2004). There is an accumulation of Igs, mainly originating from serum but also produced locally in the udder, before parturition, and then the Ig level declines rapidly during the first 2 weeks of lactation. Thus the decrease in milk PP as lactation proceeds can be expected. Similar changes in the levels of antibodies against *Helicobacter pylori* in milk of immunized cows have been observed (Korhonen et al., 1995). The significant decrease in milk PP some months after calving can also be a result of the dilution effect of the increased milk production.

All cows included in our study were antibody positive either in milk or in serum at the first two samplings, using the cut-off of the manufacturer's instruction, and were thus considered *N. caninum*-infected (Björkman et al., 1997; Varcasia et al., 2006). However, it is unknown whether they were acutely or chronically infected. Although we found considerable variation in milk PP all cows were positive throughout

the study, indicating that the cut-off used might be applicable to establish *N. caninum* status of milking cows at various stages of lactation.

Changes in the serum PP found in here were not consistent with previous studies that have reported fluctuation of the levels of *N. caninum* antibodies in serum as the result of a reactivation of parasite infection in chronically infected animals (Paré et al., 1997; Stenlund et al., 1999; Williams et al., 2003). A possible reason for the constant level of serum PP within lactation for our cows might be a weak reactivation of the parasite. Because all the cows in this study were housed in the same building as their herd mates, we assumed that the potential risk of reinfection of *N. caninum* should be constant over time in each herd.

Noticeably the milk PP of one cow in our study was above the cut-off at all sampling whereas the serum PP was usually considered negative throughout the study. It is not easy to explain what factors affect the levels of *N. caninum* antibodies in serum and milk of such a cow. One reason may be that, physiologically, only 30% of IgG in bovine milk is derived from serum whereas most milk IgG is produced locally (Tizard, 2004). A study has reported that subclinical mastitis, where a high somatic cell count may be the only visible sign, can occasionally cause false positive reactions in a milk ELISA for detection of antibodies to bovine leukemia virus (Klintevall et al., 1991). In addition, Caffin et al. (1983) found that quarters infected by *Staphylococcus aureus* had increased levels of IgG1 whereas no differences in IgG1 concentrations in uninfected quarters were observed. In this study we did not test any of the cows for subclinical mastitis.

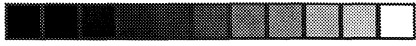
The association between the serum and milk PP supports previous results by, e.g. Ooi et al. (2000). The adjusted mean of milk PP of cows classed in PP_SERUM ≥ 55 was significantly higher than that of cows classed in lower serum PP groups. This finding supports previous reports that individual milk samples of lactating cows can be used as alternative materials for either screening or diagnosis of *N. caninum* infection (Schaes et al., 2004; Varcasia et al., 2006). The use of milk samples is not only simpler and non-invasive for animals than blood sampling, but also more cost-effective since the farmers themselves can collect the milk sample and deliver to the laboratory.

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Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand

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Abstract

The relationship between the level of *Neospora caninum* antibodies in bulk milk and the seroprevalence in lactating cows was investigated. Bulk milk was also used to estimate the prevalence of *N. caninum* infection in dairy herds in the northeast and north Thailand. Bulk milk and individual serum from all lactating cows in 11 herds as well as 220 bulk milk samples from nine milk collection centres were analysed for presence of *N. caninum* antibodies using an iscom ELISA. In the 11 herds the bulk milk absorbances ranged between 0.04 and 0.89 and the seroprevalences varied between 0 and 46%. Five herds had milk absorbances below 0.20, among those were the two herds housing only seronegative lactating cows. In the remaining three herds with such low bulk milk absorbances one or two cows (5–14%) were seropositive. Six of the investigated herds had bulk milk absorbances above 0.20. In the two herds with the highest bulk milk absorbances more than 30% of the cows were seropositive. Using an absorbance of 0.20 to discriminate between negative and positive herds, 102 (46%) of 220 bulk milk samples were judged positive. There was no significant difference in mean bulk milk absorbance between the milk collection centres within each region. However, the proportion of herds with bulk milk absorbances ≥ 0.50 in the north was statistically ($P < 0.01$) higher than that in the northeast. It was concluded that bulk milk antibody testing can be used to identify *N. caninum*-infected herds and that *N. caninum* is a common infection in dairy herds in Thailand. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cattle-protozoa; *Neospora caninum*; Iscom ELISA; Bulk milk; Prevalence; Thailand

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1. Introduction

Neospora caninum is a cyst-forming coccidian parasite which was first detected in Norwegian dogs (Bjerkås et al., 1984), and was described and named in 1988 (Dubey et al., 1988). The parasite has later been found in several animal species including cattle and other domestic and wild ruminants (Dubey and Lindsay, 1996). Bovine *N. caninum* infection has a worldwide distribution (Dubey, 1999; Hemphill and Gottstein, 2000) and has recently been reported in the southeast Asian countries of Vietnam (Huong et al., 1998) and Thailand (Suteeraparp et al., 1999; Kashiwazaki et al., 2001). *N. caninum* is considered as a major cause of bovine abortion in several countries (Anderson et al., 1991; Atkinson et al., 2000; Hemphill and Gottstein, 2000). The most important route of infection in cattle is transplacental transmission (Björkman et al., 1996; Paré et al., 1996; Hietala and Thurmond, 1999; Davison et al., 1999b). Horizontal transmission also occurs, and cattle can be infected by ingestion of *N. caninum* oocysts shed in the faeces of dogs, a natural definitive host for the parasite (McAllister et al., 1998; Basso et al., 2001). Cattle can probably also be infected by eating tissues, e.g. placentae, containing parasites. The infection usually has a chronic course and parasites appear to persist throughout the life of an infected animal (Björkman et al., 1996).

Presence of antibodies to *N. caninum* indicates that an animal is infected with the parasite and a number of assays for demonstration of specific antibodies have been developed (Björkman and Uggla, 1999). The *N. caninum* iscom ELISA is such a test that has been used to detect antibodies in bovine serum, foetal fluid and milk (Björkman et al., 1997; Slotved et al., 1999), and is likely to be applicable also on bulk milk (Björkman and Lundén, 1998).

Bulk milk antibody testing is routinely being applied to measure antibodies to several infectious agents such as bovine viral diarrhoea virus (BVDV), bovine viral leucosis, and infectious bovine rhinotracheitis virus/bovine herpesvirus 1 (Pritchard, 2001). Analysis of bulk milk has been used to estimate the prevalence of viral diseases in British cattle (Paton et al., 1998), and is used in the ongoing control programme for BVDV in Scandinavia (Lindberg and Alenius, 1999).

In the present study, we investigated the relationship between the level of *N. caninum* antibodies in bulk milk and the seroprevalence in lactating cows in Thai dairy herds. Bulk milk samples were then used in a survey to estimate the prevalence of *N. caninum* infection in dairy herds in the northeast and the north of Thailand.

2. Materials and methods

2.1. Samples

The study includes bulk milk and individual serum from all lactating cows in 11 herds as well as 220 bulk milk samples from nine milk collection centres in the northeast and the north regions of Thailand. The location of the herds is shown in Fig. 1.

Between May and August 2000, 151 bulk milk samples were randomly collected at seven milk collection centres located in three provinces in northeast Thailand; Khon Kaen, Udorn Thani, and Sakon Nakorn. Sixty-nine bulk milk samples were also collected at

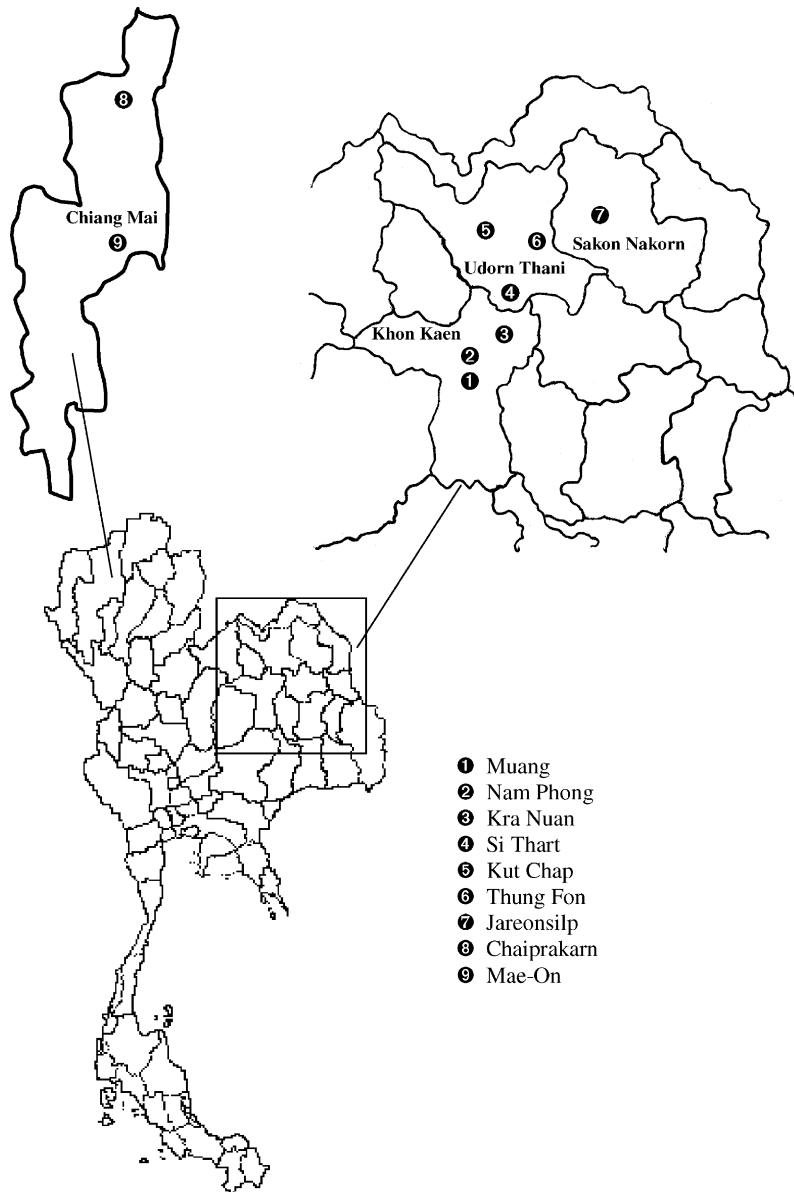


Fig. 1. Map of Thailand showing the location of the nine milk collection centres in four provinces from which bulk milk samples were collected.

two milk collection centres in Chiang Mai province in the north during January to June 2001.

To study the relationship between presence of *N. caninum*-specific antibodies in the bulk milk and prevalence of seropositive lactating cows in the herd, 11 dairy herds in the Khon Kaen province were investigated in detail. The total number of lactating cows in these herds ranged from 10 to 19. The farms were visited in August 2001, when individual serum samples were collected from all lactating cows. The blood samples were collected from the coccygeal vein into sterile tubes without additives. A sample from the bulk milk was also taken from each herd. Information was obtained from the owners regarding how many, and which, of the cows that were lactating at the time of sampling.

2.2. Sample treatment and storage

The bulk milk samples collected at the milk collection centres and on the farms were immediately stored at +4 to +8 °C and delivered to the laboratory on the same day. The blood samples were kept at room temperature (approximately +30 °C) for a maximum of 4 h. Both clotted blood and bulk milk samples were centrifuged at 1000 × *g* for 15 min at +4 °C. The serum was removed and the skimmed milk was collected. The sera and skimmed milk were inactivated at +56 °C for 30 min and stored at –20 °C until analysis.

2.3. Serological analysis

All samples were analysed for presence of antibodies to *N. caninum* using an iscom ELISA as described by Björkman et al. (1997). The serum and bulk milk samples were diluted 1:100 and 1:2, respectively, in PBS-Tween before analysis. Sera from non-infected

Table 1
N. caninum antibody levels in bulk milk and serum samples from all lactating cows in 11 dairy herds in the Khon Kaen province, northeast Thailand

Herd number	Number of seropositive lactating cows/total number of lactating cows (%)	Antibody level of bulk milk sample	Antibody levels of seropositive lactating cows
1	0/12 (0)	0.04	–
2	1/17 (6)	0.06	0.26
3	0/18 (0)	0.09	–
4	2/14 (14)	0.09	0.21, 0.28
5	1/19 (5)	0.12	0.93
6	1/14 (7)	0.22	0.79
7	2/19 (11)	0.22	0.64, 0.81
8	5/12 (42)	0.31	0.23, 0.24, 0.31, 0.77, 0.78
9	1/10 (10)	0.42	0.92
10	6/13 (46)	0.85	0.72, 0.76, 0.79, 0.79, 0.90, 0.95
11	5/16 (31)	0.89	0.52, 0.59, 0.79, 0.80, 0.95
Total	24/164 (15)		

An *N. caninum* iscom ELISA was used for demonstration of antibodies, and results are given as absorbance values. Absorbance values ≥ 0.20 were considered positive.

and experimentally *N. caninum*-infected calves were included in each assay as controls. All absorbance values were correlated to a positive control serum with a mean absorbance value of 1.00. Sera with corrected absorbances ≥ 0.20 were considered positive (Björkman et al., 1997).

2.4. Statistical analysis

Differences in mean bulk milk absorbance between the milk collection centres and the regions were analysed by analysis of variance (ANOVA). The two-sample test for proportions was used to compare the number of bulk milk samples in different classes between regions.

3. Results

When bulk milk samples together with individual serum samples from all the lactating cows from 11 dairy herds were analysed for presence of *N. caninum* antibodies, the bulk milk absorbances were found to range between 0.04 and 0.89, and the seroprevalences in the herds varied between 0 and 46% (Table 1). Five herds had a bulk milk absorbance < 0.20 , and among these were two herds housing only seronegative lactating cows. In the remaining three herds with low bulk milk absorbances one or two cows were seropositive giving seroprevalences of 5–14%. Six of the investigated herds had bulk milk absorbances above 0.20, and in these herds the seroprevalences were 7–46%. In the two herds with the

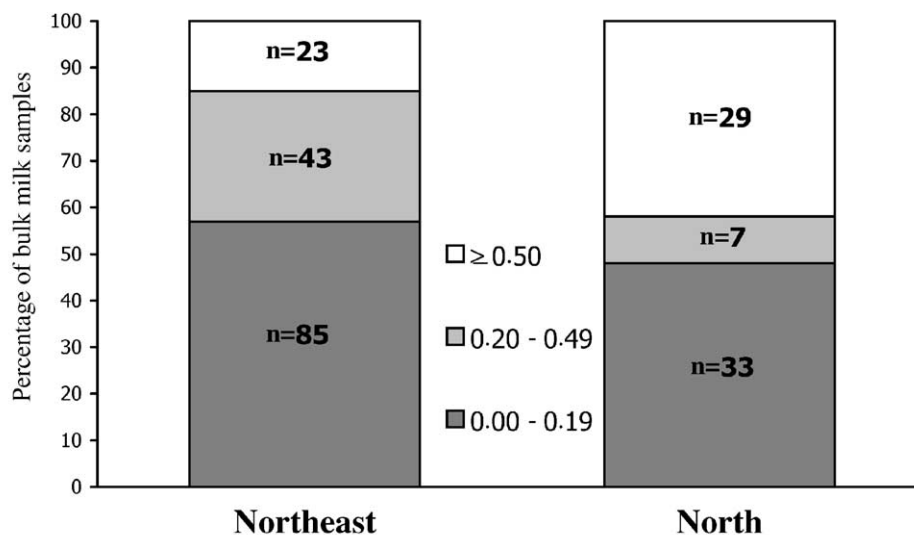


Fig. 2. Distribution of bulk milk samples based on *N. caninum* antibody levels. Presence of antibodies was demonstrated by *N. caninum* iscom ELISA, and results are given as absorbance values. The samples were collected from northeast ($n = 151$) and north ($n = 69$) Thailand.

Table 2
Results from *N. caninum* antibody analysis of 220 bulk milk samples collected at nine milk collection centres in northeast and north Thailand

Milk collection centre	North								
	1	2	3	4	5	6	7	8	9
Number of herds	11	40	24	20	20	19	17	25	44
Number of herds with absorbance ≥ 0.20 (%)	6 (55)	18 (45)	11 (46)	11 (55)	5 (25)	10 (53)	5 (29)	13 (52)	23 (52)
Mean absorbance	0.37	0.22	0.27	0.28	0.23	0.37	0.19	0.36	0.46
Median absorbance	0.20	0.17	0.18	0.26	0.10	0.28	0.16	0.20	0.29
Absorbance range	0.09–0.90	0.03–0.72	0.05–0.89	0.04–0.80	0.04–0.89	0.01–0.94	0.07–0.37	0.06–0.92	0.04–1.08

Presence of antibodies was demonstrated by *N. caninum* iscom ELISA, and results are given as absorbance values. Absorbance values ≥ 0.20 were considered positive.

highest bulk milk absorbances (i.e. 0.85 and 0.89, respectively), more than 30% of the cows were seropositive.

The serum antibody levels in the seropositive cows varied considerably. In herd 4, with 14% seroprevalence and a bulk milk absorbance of 0.09, both the seropositive cows had fairly low serum absorbance values (0.21–0.28), whereas the cows in the four herds (herds 5, 6, 7 and 9) with lower seroprevalences together with a higher bulk milk absorbance compared with herd 4, all had high serum absorbances (≥ 0.64).

One hundred and two (46%) and 52 (24%) of 220 bulk milk samples collected from northeast and north Thailand had absorbances ≥ 0.20 and ≥ 0.50 , respectively (Fig. 2). The number of bulk milk samples and the absorbance distributions in each milk collection centre are summarised in Table 2. Using an absorbance value of 0.20 to discriminate between negative and positive herds, 66 (43%) and 36 (52%) herds in the northeast and the north, respectively, were judged positive. There was no statistically significant difference in the proportion of positive herds between the two regions. However, the proportion of bulk milk samples with absorbances ≥ 0.50 was significantly ($P < 0.01$) higher in the north than in the northeast. Comparing the mean absorbances, there was no significant difference between the milk collection centres within each region. However, the mean absorbance in the bulk milk samples from the north was significantly ($P < 0.05$) higher than in those from the northeast.

4. Discussion

It has previously been shown that bulk milk can be used to detect antibodies to infectious agents such as viruses (Niskanen et al., 1991; Elvander et al., 1995; Pritchard, 2001; Armstrong and Mathew, 2001), bacteria (Vanzini et al., 2001; Veling et al., 2001) and nematode parasites (Kloosterman et al., 1993). Bulk milk antibody testing is a useful and inexpensive method for diagnosis of infectious diseases in dairy herds. The samples can easily be collected and the herds investigated repeatedly. It is a simpler method than individual serum sampling and is also harmless to the animals. Additionally, the farmers themselves can collect and submit samples to the laboratory for testing. Today, bulk milk analysis is routinely used as a tool in diagnosis and prophylaxis of BVDV infections in dairy herds in Scandinavia (Niskanen et al., 1991; Lindberg and Alenius, 1999).

In this study, the *N. caninum* iscom ELISA was used to demonstrate presence of antibodies to the parasite. When the relationship between antibody levels in serum from the individual cows and the bulk milk was measured in 11 herds, a low bulk milk absorbance was found in the two herds in which all cows were seronegative to *N. caninum*. Further, the two herds that had the highest absorbances in the bulk milk both had a high seroprevalence, i.e. 30 and 46%, among the lactating cows. However, there was no direct association between seroprevalence and bulk milk absorbance. These results are not fully consistent with previous studies that report a high correlation between BVDV antibodies in the bulk milk and prevalence of antibody-positive cows (Niskanen et al., 1991; Niskanen, 1993). A probable reason for this could be that not only the proportion of infected cows but also their antibody levels, lactation stage, and milk yield influence the bulk milk absorbance. This is likely to be more important in small herds with few cows contributing to the bulk milk. For example, in the

present study comprising herds with 10–19 lactating cows, the bulk absorbances varied considerably among the four herds that had only one single seropositive cow.

The results presented here are consistent with previous reports that *N. caninum* antibodies were detectable in the bulk milk when at least 10–15% of the lactating cows were seropositive (Björkman and Lundén, 1998; Ugglä et al., 2000). Our results show that bulk milk can be used to identify *N. caninum*-infected herds. Further, they suggest that a bulk milk absorbance of ≥ 0.50 indicates that the herd has high prevalence of *N. caninum*-infected cows. However, it is worth noting that a low bulk milk absorbance does not exclude the presence of infection in the herd. Further validations for truly negative herds are still needed in order to establish the stability of the test and an accurate cut-off value.

Based on the investigation in the 11 herds, an absorbance value of 0.20 was used to discriminate between negative and positive herds. Forty-six percent of the dairy herds in the investigated areas were infected with *N. caninum*. Additionally, the mean bulk milk absorbance of dairy herds in the north was higher than in the northeast, and the north had higher proportion of herds with a bulk milk absorbance ≥ 0.50 . Seroprevalences of 12–70% in dairy cattle have previously been reported in northeast Thailand by IFAT at different cut-off levels (Suteeraparp et al., 1999; Kashiwazaki et al., 2001). A considerably lower seroprevalence, 6%, was reported from the central region of Thailand (Suteeraparp et al., 1999) and from the neighbouring country Vietnam (Huong et al., 1998). Together, these results show that a high proportion of Thai dairy herds harbour *N. caninum*-infected cows and that cattle in the northern part of the country have a higher risk of being infected compared with those in the northeast. The reason for this difference between the regions is not known.

In the present study, we found a large variation in seroprevalence among the investigated herds, and in some of them more than 30% of the cows were infected with *N. caninum*. This variation is similar to what has been found in dairy herds by others (Thurmond et al., 1997; Davison et al., 1999a; Dijkstra et al., 2001; Pitel et al., 2001). To date, little information on the impact of *N. caninum* to the Thai livestock is available. However, since such a high proportion of the dairy herds harbour infection it can be expected that neosporosis may cause bovine abortion in Thailand as in other parts of the world. Indeed, in a previous study, Suteeraparp et al. (1999) found an association between *N. caninum* seropositivity and bovine abortion in one of three investigated areas. In this area, there was also a rapid increase in seroprevalence, possibly as a result of postnatal infection (Suteeraparp et al., 1999; Kashiwazaki et al., 2001). Due to the design of the present study it was not possible to link the presence of antibodies to *N. caninum* in the bulk milk to abortion. However, as the infection seems to be widespread in Thailand its importance for reproductive performance deserves further attention.

Acknowledgements

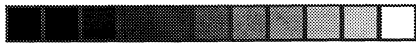
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III





Application of repeated bulk milk testing for identification of infection dynamics of *Neospora caninum* in Thai dairy herds

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Abstract

In this prospective study, repeated bulk milk tests were evaluated to establish the *Neospora caninum* antibody status and to describe infection dynamics and changes in prevalence of *N. caninum* in Thai dairy herds. Bulk milk from 418 herds in northeast Thailand were collected at three consecutive samplings, in December 2002 (sampling 1), April (sampling 2) and December 2003 (sampling 3). All samples were analysed for presence of *N. caninum* antibodies by iscom ELISA, and the optical density values were dichotomized at three different cut-offs. Herd status at either sampling 1 or sampling 2 was used to predict herd status at sampling 3. Changes in both sensitivity and specificity at the first samplings at all cut-offs were consistent with commonly seen patterns when evaluating performance of diagnostic tests. In addition, the predictive positive values (PPV) of herd status at each of the two samplings were more affected by time of sampling than choice of cut-off, whereas the predictive negative values (PNV) increased with increasing cut-off. Herd status at the first samplings was also interpreted in combination, i.e. herds that were negative at both samplings were considered negative and positive otherwise in Combination A, while herds positive at both samplings were considered positive in Combination B. When using these combinations, Combination A gave higher PNV but lower PPV than Combination B at all cut-offs. Using the optical density value 0.20 as cut-off to establish the herd *N. caninum* antibody status, 270 herds (65%) kept their status throughout the study period, but 148 herds converted their status at least once. Interestingly, 136 (86%) of the 158 herds that were considered negative at sampling 1 and sampling 2 remained negative at sampling 3. In addition of the 159 herds considered positive at the first two samplings, 134 (84%) were still positive at sampling 3. We concluded that repeated bulk milk testing at regular intervals provided better information about herd *N. caninum* status

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than a single test. Further, our results showed that the infection is prevalent in northeast Thailand, but that a herd can keep a negative infection status despite the frequent presence of dogs.

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Keywords: *Neospora caninum*; Iscom ELISA; Bulk milk; Herd status; Dairy cattle; Thailand

1. Introduction

The protozoan *Neospora caninum* is an intracellular parasite that is well-recognized as a major cause of bovine abortion world-wide (Dubey, 2003) and leads to substantial economic losses in countries with intensive cattle production (Thurmond and Hietala, 1996; Dubey, 1999; Trees et al., 1999; Waldner et al., 2001). Common clinical manifestations include abortion, stillbirth and birth of weak calves (Dubey, 2003). Further, infected cows are likely to give birth to congenitally infected but clinically normal calves (Pare et al., 1996; Williams et al., 2000), which seem to carry the infection for life (Björkman et al., 1996).

Thailand has a total dairy cattle population of about 0.38 million (DLD, 2004). Most dairy herds are owned by smallholder farmers and comprise 10–15 milking cows. These herds are established nearby or within a village and some are located close to other farms. Dairy cattle are normally housed in open buildings and fed roughage and concentrate. Some herds are allowed to graze even though fresh grass is usually cut, carried and given to the cattle during the rainy season. In central Thailand 6% of dairy cattle have been reported to be seropositive to *N. caninum* (Suteeraparp et al., 1999; Kyaw et al., 2004), whereas in northeast Thailand higher infection rates, 12–70%, have been found (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002). However, little is known about the dynamics of *N. caninum* infection and changes in the infection rate in Thai dairy herds.

In cattle, antibody assays are widely used to diagnose exposure to *N. caninum*, and different tests have been developed for demonstration of *N. caninum* antibodies in serum, milk from individual cows and bulk milk (Björkman and Uggla, 1999; Jenkins et al., 2002). Bulk milk testing has advantages over analysis of individual serum and milk since it is rapid and cost-effective. Analysis of bulk milk has been widely used for demonstration of antibodies to a variety of agents

such as viruses (Emanuelson et al., 1989; Niskanen et al., 1991; Lindberg and Alenius, 1999), bacteria (Barling et al., 2000; Nielsen et al., 2000) and parasites (Sanchez et al., 2004; Schares et al., 2004a) in cattle, but is not yet commonly used for detection of *N. caninum* infection. When an *N. caninum* iscom ELISA was evaluated for application on bulk milk, the optical density (OD) values were related to the within-herd prevalence (Chanlun et al., 2002; Frössling, 2004). However, neither the sensitivity nor the specificity of a single test to determine whether a herd was infected or not, was 100% (Frössling, 2004). Repeated bulk milk testing might be one way to increase the predictability of a herd's true *N. caninum* status.

The objective of this study was firstly to evaluate the application of repeated bulk milk sampling to establish the *N. caninum* antibody status of dairy herds. We further aimed at describing infection dynamics and changes in prevalence of *N. caninum* in Thai dairy herds based on bulk milk antibody analysis.

2. Materials and methods

2.1. Study design

A prospective study was carried out at seven milk collection centres: Muang, Nam Phong, Kra Nuan, Si Thart, Kut Chap, Thung Fon and Jareonsilp, in northeast Thailand. The location of the target milk collection centres was described in Chanlun et al. (2002). Out of 704 dairy herds, 418 that delivered milk to the seven milk collection centres were randomly selected. Between 5 and 15 milking cows contributed to the bulk milk in each herd.

To evaluate the performance of repeated bulk milk antibody test, bulk milk samples of all 418 dairy herds were collected at three consecutive occasions: December 2002 (sampling 1), April 2003 (sampling

2) and December 2003 (sampling 3). All samples were collected by the same person. The numbers of samples collected at each milk collection centre are shown in Table 1.

2.2. Sample treatment and storage

Bulk milk samples (20 ml/herd) were aseptically collected, immediately put into iceboxes (+4 to +8 °C) and delivered to the laboratory on the same day. All samples were centrifuged at 1000 × *g* for 15 min at +4 °C. Skim milk was collected, inactivated at 56 °C for 90 min and stored at –20 °C until analysis.

2.3. Antibody detection

An iscom ELISA was used to detect presence of *N. caninum* antibodies (Björkman et al., 1997). The milk samples were diluted 1:2, in PBS-Tween before analysis. Sera collected from non-infected and experimentally infected calves, heat-treated in the same manner as the milk samples and diluted 1:100, were included in each test series as controls. All samples were analyzed in duplicates and the OD was measured at 450 nm. Mean OD value of the duplicate was calculated and subsequently correlated to the positive control serum, which had a mean OD value of 1.0.

2.4. Data analysis

The bulk milk OD was dichotomized at three different cut-off levels, i.e. 0.10, 0.15 and 0.20, in order to distinguish *N. caninum* antibody status of

herds as negative or positive. The cut-off values were based on a preliminary study that was carried out to evaluate the iscom ELISA for use on bulk milk (Frössling, 2004). A test result was considered positive if the OD was higher than or equal to the particular cut-off level, and negative otherwise.

The results at sampling 1 and sampling 2 were used to predict herd antibody status at sampling 3 (reference result). The predictive abilities were determined using methods commonly applied in evaluation of diagnostic tests, i.e. the sensitivity (Se), specificity (Sp), predictive negative value (PNV) and predictive positive (PPV) value, were calculated. The Se of a diagnostic test is defined as the probability that the test is positive, given that the “true” status is positive. The Sp of a test is defined as the probability that the test is negative, given that the “true” status is negative. The PNV of a test is defined as the probability that the “true” status is negative, given that the test is negative. The PPV of a test is defined as the probability that the “true” status is positive, given that the test is positive. (Thrusfield, 1995).

When several test results are available they can be interpreted in combination to either increase the PNV (termed “parallel” interpretation) or PPV (termed “serial” interpretation) (Thrusfield, 1995). We therefore created a combined test result based on the test results at sampling 1 and sampling 2. In Combination A, a herd was considered negative if the status at both sampling 1 and sampling 2 was considered negative, and positive otherwise, i.e. parallel interpretation. In Combination B, a herd was considered positive if a herd status at both sampling 1 and sampling 2 was

Table 1
Results from *N. caninum* antibody analysis of 418 bulk milk samples collected at seven milk collection centres in Thailand

	Milk collection centre	Total number of herds	Sampling 1 ^a		Sampling 2 ^a		Sampling 3 ^a	
			Median OD	OD range	Median OD	OD range	Median OD	OD range
1	Muang	170	0.27	0.00–1.00	0.25	–0.03–1.11	0.22	0.02–1.34
2	Nam Phong	130	0.20	0.07–0.90	0.11	0.01–1.16	0.11	0.00–1.31
3	Kra Nuan	25	0.07	0.00–0.87	0.10	0.04–0.95	0.10	0.05–0.91
4	Si Thart	31	0.24	0.04–0.79	0.14	0.06–1.02	0.23	0.07–1.53
5	Thung Fon	20	0.24	0.04–1.08	0.14	0.09–0.98	0.11	0.01–1.39
6	Kut Chap	25	0.22	0.10–0.82	0.16	0.06–0.92	0.20	0.04–1.19
7	Jaroensilp	17	0.24	0.14–0.88	0.14	0.08–0.80	0.16	0.05–1.45

The *N. caninum* iscom ELISA was used for demonstration of antibodies, and the results are given as optical density (OD) values.

^a Sampling 1, 2 and 3 were carried out in December 2002, April 2003 and December 2003, respectively.

considered positive, and negative otherwise, i.e. serial interpretation.

Estimates and 95% confidence intervals of Se, Sp, PPV and PNV were calculated using WinEpiscope software version 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield: <http://www.zod.wau.nl/qve>).

3. Results

OD values varied considerably within each of the seven milk collection centres and at the three different samplings (Table 1). Kra Nuan had the lowest median OD (≤ 0.10), whereas Muang had median OD ≥ 0.22 at all samplings.

3.1. Characteristics of bulk milk antibody test

When using a single herd status, i.e. either sampling 1 or sampling 2, to predict herd status at sampling 3, sampling 1 gave higher estimates of Se, but lower Sp than sampling 2 (Table 2). It was

Table 2
Estimates and 95% confidence interval (CI) of the sensitivities (Se), specificities (Sp), predictive positive (PPV) and negative (PNV) values at three different cut-offs when using a herd status at sampling 1^a and sampling 2^a to predict a status at sampling 3^a of 418 dairy herds

Bulk milk cut-off		Sampling 1		Sampling 2	
		Point estimate	95% CI	Point estimate	95% CI
0.10	Se	94	91–97	82	78–87
	Sp	16	10–22	57	49–65
	PPV	67	62–71	77	72–82
	PNV	60	45–75	64	56–73
0.15	Se	87	83–92	75	70–81
	Sp	43	36–50	76	70–82
	PPV	63	58–68	78	72–83
	PNV	75	67–83	74	67–80
0.20	Se	84	78–89	74	68–80
	Sp	67	61–73	83	78–87
	PPV	69	63–75	79	73–85
	PNV	82	77–88	78	73–84

A bulk milk cut-off was based on the optical density of the *N. caninum* iscom ELISA.

^a A bulk milk of sampling 1, 2 and 3 were carried out in December 2002, April 2003 and December 2003, respectively.

noticeable that the estimate of PPV was more affected by sampling occasion than choice of cut-off. In addition, the estimated PPV at sampling 2 was usually higher than those values at sampling 1 for all the three cut-off levels. In contrast, PNV of a herd status at both sampling 1 and sampling 2 increased with increasing cut-off, whereas differences in the estimated PNV between both samplings at the same cut-off did not appear to differ.

Table 3 shows the estimates of PPV and PNV of a herd status using Combination A and Combination B to predict a herd status at sampling 3. The estimated PPV of Combination B for all the three cut-offs were higher than the PPV of Combination A, but the estimated PPV of both Combination A and Combination B appeared to be similar among the different cut-offs. On the other hand, the estimated PNV of Combination B at all cut-offs were lower than the PNV of Combination A. Further, the estimated PNV of both Combination A and Combination B were consistently higher when changing to a higher cut-off.

3.2. Dynamics of *N. caninum* infection

The distribution of herds according to herd status at the three samplings, using the OD value 0.20 as cut-off

Table 3
Predictive positive (PPV) and negative (PNV) values and 95% confidence interval (CI) at three different cut-offs predicting a herd status at sampling 3^a using two combinations of status at sampling 1 and sampling 2 of 418 dairy herds

Bulk milk cut-off		Combination A ^b		Combination B ^c	
		Point estimate	95% CI	Point estimate	95% CI
0.10	PPV	66	62–71	78	74–83
	PNV	74	56–92	62	54–70
0.15	PPV	63	57–68	80	74–85
	PNV	84	76–91	70	64–76
0.20	PPV	67	61–72	84	77–90
	PNV	86	81–91	76	71–82

A cut-off was based on the optical density of the iscom ELISA for demonstration the presence of *N. caninum* antibodies.

^a A herd was considered positive if the bulk milk OD at sampling 3 was greater or equal to the particular cut-off, and considered negative otherwise.

^b A herd was considered negative if both the status at sampling 1 and sampling 2 were negative, and considered positive otherwise.

^c A herd was considered positive if both the status at sampling 1 and sampling 2 were positive, and considered negative otherwise.

Table 4
Neospora caninum herd status at three consecutive samplings using an iscom ELISA for demonstration the presence of antibodies

Sampling 1 ^a	Sampling 2 ^a	Sampling 3 ^a		Total
		Negative	Positive	
Negative	Negative	136	22	158
Negative	Positive	14	10	24
Positive	Negative	48	29	77
Positive	Positive	25	134	159
Total		223	195	418

A herd with a bulk milk optical density ≥ 0.20 was considered positive, and considered negative otherwise.

^a A bulk milk of sampling 1, 2 and 3 were carried out in December 2002, April 2003 and December 2003, respectively.

to establish the herd *N. caninum* antibody status, is given in Table 4. Of the 418 herds, 270 (64.6%) were considered of the same status throughout all three samplings whereas the remaining 148 (35.4%) herds changed their status at least once. Interestingly, of the 158 herds considered negative at both sampling 1 and sampling 2, 136 (86.1%) remained negative at sampling 3. In addition, 134 (84.3%) out of the 159 herds considered positive at sampling 1 and sampling 2 were positive at the last sampling.

Overall, the proportions of the herds considered positive at sampling 1, sampling 2 and sampling 3 were 56.5%, 43.8% and 46.7%, respectively. The number of positive herds at the different samplings at each milk collection centre is shown in Table 5. Kra Nuan had a consistently low prevalence as compared with the other milk collection centres. It is also noticeable that the proportion of *N. caninum* antibody

positive herds was lower at sampling 3 than at sampling 1 in all but one of the milk collection centres.

4. Discussion

When results of a single sampling were used to predict a herd status at sampling 3 we found lower Se and higher Sp at both sampling 1 and sampling 2 when changing to a higher cut-off. These changes in both the estimated Se and Sp with increasing cut-off are consistent with what can be expected when evaluating the performance of diagnostic tests (Thrusfield, 1995; Rothman, 2002). The estimates of both Se and Sp were also affected by the sampling occasion, which emphasizes the importance of sampling interval. The fact that the Se at sampling 2 was lower than at sampling 1 was unexpected and cannot easily be explained since both higher Se and Sp would be expected at the sampling occasion closest to sampling 3.

Combination A and Combination B together improved the estimated predictive values compared to using either the results of sampling 1 or sampling 2 alone. Combination A increased the estimated PNV and can thus be used to confirm absence of *N. caninum* infection. In contrast, Combination B improved the point estimate of PPV and is useful to confirm infection. However, neither PPV nor PNV is completely reliable, so there is always a risk of misclassification. Further, both PPV and PNV have some shortcomings in terms of evaluating test characteristics because they are greatly affected by

Table 5
 Number (percentage) of *Neospora caninum* positive herd in seven milk collection centres at three sampling occasions

	Milk collection centre	Total number of herds	Number of positive herds (%)		
			Sampling 1 ^a	Sampling 2 ^a	Sampling 3 ^a
1	Muang	170	105 (62)	91 (54)	90 (53)
2	Nam Phong	130	64 (49)	45 (35)	52 (40)
3	Kra Nuan	25	7 (28)	9 (36)	7 (28)
4	Si Thart	31	21 (68)	13 (42)	19 (61)
5	Thung Fon	20	10 (50)	9 (45)	8 (40)
6	Kut Chap	25	16 (64)	11 (44)	13 (52)
7	Jaroensilp	17	12 (71)	4 (24)	6 (35)

Total number of herds, 418. Presence of *N. caninum* antibodies was demonstrated using an iscom ELISA. A herd with a bulk-milk optical density ≥ 0.20 was considered positive.

^a A bulk milk of sampling 1, 2 and 3 were carried out in December 2002, April 2003, and December 2003, respectively.

the prevalence in the study population (Thrusfield, 1995). It is worth noting that the present study was carried out in Thailand, and that the overall seroprevalence in this region has been reported high (12–70%) (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002). The results are therefore not directly applicable to countries with relative low prevalences, such as in Sweden (Björkman et al., 2000).

When the iscom ELISA was previously evaluated for use on bulk milk, 0.20 was suggested as useful cut-off for identifying infected herds (Chanlun et al., 2002; Frössling, 2004). Using this cut-off, 134 (84%) of the 159 herds that were classified positive at the first two samplings were also positive at sampling 3, indicating that they had antibody-positive cows throughout the study. This is in agreement with international studies that have shown that *N. caninum* infection can be kept in cattle herds for a long period by repeated vertical transmission throughout pregnancies (Björkman et al., 1996; Schares et al., 1998; Frössling et al., 2005). Although vertical transmission is the dominating route, cattle can also be infected upon ingestion of oocysts shed by the definitive host of the parasite (McAllister et al., 1998). Dogs and coyotes are definitive hosts of *N. caninum* (McAllister et al., 1998; Lindsay et al., 1999; Gondim et al., 2004), but coyotes are not present in Thailand. Dogs are, however, common in the study area and have easy access to cattle, pasture and feed storage rooms. Other studies have shown that the presence of dogs on farms is a potential risk factor for bovine *N. caninum* infection (Bartels et al., 1999; Mainar-Jaime et al., 1999; Wouda et al., 1999; Schares et al., 2004b), but the relative importance of dogs in spread of *N. caninum* in the current population of herds is not known.

Furthermore, 136 (86%) of the 158 herds that were negative at the first two samplings remained negative, suggesting that they had only non-infected cows, or a low proportion of antibody-positive cows. The fact that so many herds kept their negative infection status shows that these Thai dairy herds avoid *N. caninum* infection in spite of dogs being frequently present. This might be because that the infection rate in dogs is low; however, no information is available about the prevalence of *N. caninum* in dogs in the study areas. Recently, though, a low (1.2%) seroprevalence of farm dogs in the central Thailand has been reported (Kyaw et al., 2004).

A change in bulk milk antibody status was observed in 35% of the herds studied. Specifically, 14% of the 158 herds considered negative at the first two samplings were positive at sampling 3. This change in antibody status was either caused by a chronically infected heifer or cow that did not contribute to the bulk milk at the first samplings but that contributed at the third sampling, or an antibody-positive cow that had been purchased between the samplings. Alternatively, a horizontal infection may have occurred after sampling 2. Surveillance of prevalence in *N. caninum* infection in dogs on farms and neighboring areas would greatly assist in determining the possible role of dogs in the epidemiology of bovine neosporosis in Thai dairy herds. On the other hand, 16% of the herds considered positive at the first two samplings were negative at sampling 3, indicating that most or all of the antibody-positive cows were removed from the milking group. They might have been either in a dry period, or have been culled from the herd, but no detailed information about culling was available. Studies have reported that *N. caninum*-infected cows have a higher risk of abortion during their pregnancies than non-infected cows (Thurmond and Hietala, 1997; Wouda et al., 1998). An aborted cow is likely to have a shorter lactation (Thurmond and Hietala, 1996) and thus spend less time in the milking group. Furthermore, infected cows might be culled because of their reproductive problems without farmers knowing that they are *N. caninum* antibody positive (Thurmond and Hietala, 1996).

The wide variation in bulk milk OD and the differences in prevalence between the seven milk collection centres (Tables 1 and 5) are in accordance with our previous study (Chanlun et al., 2002). Also, the variation seemed to be different among the milk collections centres. The existence of a high proportion of *N. caninum* antibody-positive herds confirmed that *N. caninum* infection is widespread in Thai dairy cattle populations. The prevalence of positive herds decreased slightly from sampling 1 to sampling 3, but this decrease was not due to any action taken by farmers because farmers were not informed about sampling results. A possible explanation could instead be a concurrent structural change in the dairy herds in the area implying that herd sizes decreased, whereby old and possibly problem-cows (i.e. high

risk for *N. caninum* positive) were most likely to be removed.

Results from a single bulk milk test should be interpreted cautiously since only lactating cows contribute to the herd status. A negative result of a single bulk milk test does not completely exclude the infection, but repeated bulk milk testing at regular intervals provides better information to determine herd status. However, the usefulness of the application of repeated bulk milk tests in other countries where the average number of lactating cows per herd is higher than in Thai dairy herds needs to be verified.

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IV



A longitudinal study of seroprevalence and seroconversion of *Neospora caninum* infection in dairy cattle in northeast Thailand

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Abstract

A long-term study was carried out in 11 dairy herds in the Khon Kaen province of northeast Thailand between August 2001 and November 2004. The objective was to investigate seroprevalence dynamics of *Neospora caninum* infection in the herds and to demonstrate patterns of seroconversion in individual cattle. Each herd was visited once a year, in total four times, and sera from cattle >3 months of age and farm dogs and a sample from the bulk milk were collected. All samples were analysed for presence of specific antibodies by an *N. caninum* iscom ELISA. The overall percentage of antibody-positive cattle was constant and varied only between 10-13% over the 4 years, but the variation in within-herd seroprevalence between herds was substantial. Two herds had $\geq 20\%$ seropositive animals at all samplings and consistently high bulk milk OD, whereas 2 herds had no seropositive animal at the last two samplings and low bulk milk OD. Five herds had a decreasing trend of within-herd seroprevalence whereas the remaining six herds had a higher portion of test-positive individuals at the end of the study. A total of 424 individuals were sampled more than once; 344 (81%) and 32 (8%) were consistently antibody-negative and antibody-positive, respectively. The proportions of animals that changed from being seronegative to seropositive and from being seropositive to seronegative between the years were 3.9-4.6% and 19-39%, respectively. Apparent vertical and horizontal transmission rates were 58% (95% CI; 44-71%) and 5% (95% CI; 3-7%), respectively. In conclusion, the overall percentage of *N. caninum* antibody-positive cattle was constant over the

years, but the within-herd seroprevalence varied substantially between the herds. Seroconversions were likely to occur in individual cattle although most animals had consistent serological status throughout the study.

Keywords: *Neospora caninum*, Seroprevalence, Seroconversion, Iscom ELISA, Transmission, Dairy cattle

1. Introduction

Neospora caninum is an intracellular protozoan parasite that infects dairy cattle and has been shown to be associated with bovine abortion (Dubey, 2005). Substantial economic losses to the farming industry due to *N. caninum* have been estimated (Chi et al., 2002). *N. caninum* is found worldwide and seroprevalence of infection in cattle appears to differ between herds, countries and regions, depending on type of serologic test used, cut-off level used to identify the exposure and study design. In Europe, herd seroprevalences varies considerably, from 16 to 76% (Bartels et al., 2006). Epidemiological studies have reported that *N. caninum* seropositivity of cattle in the infected herds also varies considerably, up to 87% (Dubey, 2005; Frössling et al., 2005).

In cattle, the most frequent route of *N. caninum* infection is vertical transmission and an infected dam can transmit the parasite to her offspring during consecutive pregnancies (Björkman et al., 1996; Davison et al., 1999; Frössling et al., 2005). The vertical transmission rates in dairy cattle have been reported to be over 80% (Davison et al., 1999; Hietala and Thurmond, 1999), but transmission rates below 60% have also been reported (Mainar-Jaime et al., 1999; Bergeron et al., 2000; Pan et al., 2004). Horizontal acquisition has experimentally been verified to occur showing that cattle may be infected when they consume oocysts (Gondim et al., 2004a) shed by dogs or coyotes, which are the definitive hosts of the parasite (McAllister et al., 1998; Gondim et al., 2004b). Dogs become infected and shed the oocysts in faeces within two weeks after consuming tissues (Gondim et al., 2002), or placenta (Dijkstra et al., 2001) from cattle infected with *N. caninum*.

Presence of *N. caninum*-specific antibodies indicates that the individual animal has been or is infected. Currently, detection of antibodies is the only method suitable for studies designed to estimate prevalence of *N. caninum* infection in cattle populations (Björkman and Uggla, 1999).

Changes in herd and within-herd seroprevalence of *N. caninum* infection in dairy cattle have been investigated in several countries. In Thailand, a few studies have reported a considerable variation of *N. caninum* prevalence between dairy farming areas (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002; Chanlun et al., 2006). Moreover, a longitudinal study using repeated bulk milk testing has indicated that the *N. caninum* infection rates in Thai dairy herds differ both temporally and spatially (Chanlun et al., 2006). However, limited information is available about changes in prevalence of *N. caninum* infection within dairy herds and whether there is a particular pattern of serological status of

cattle that are sampled repeatedly. The objective of this study was to investigate overall seroprevalence dynamics of *N. caninum* infection in Thai dairy herds. We further aimed at demonstrating patterns of seroconversion in individual cattle.

2. Materials and methods

2.1 Study design

A long-term study was carried out in 11 dairy herds in the Khon Kaen province of northeast Thailand between August 2001 and November 2004. The herds were selected and included in the study because they had at least one *N. caninum* antibody-positive individual at the first sampling (Chanlun et al., 2002). Each herd was visited once a year; in total 4 times. Of the 11 herds, 7 herds (Herd 1, 2, 3, 4, 6, 7, and 9) were located close to other dairy farms, established nearby or within a village whereas the other 4 were more isolated (>1 kilometer from nearest farm). The cattle were of Holstein Friesian crossbreed and were normally housed in open buildings. The newborn calves were separated from their dams immediately after birth and usually given the first colostrum milked from their dams within a few hours. The calves were then kept in restricted pens until weaning at about 2 months of age when they were transferred to the area for young stock. Heifers and cows were kept together and fed roughage and concentrate, and all were allowed to graze. The farmers were given background information about the parasite and its consequences, but no specific recommendation or advice to cull test-positive animals was given. No test results were given to the farmers. All but one farm (Herd 7) had dogs, but cattle in all herds could have direct or indirect contact with stray dogs.

2.2 Sample collection, treatment and storage

Blood samples were collected from the coccygeal vein of most cattle >3 months of age at each farm visit. All samples were collected into a sterile tube and kept at the ambient temperature (approximately +30 °C) for 2-4 h. At the same time, samples from the bulk milk were collected and immediately put into an ice box (+4 to +8 °C). When farm dogs were present during the visit they were also sampled (n=36). However, no dog was sampled in Herd 2. All samples were delivered to the laboratory the same day to be centrifuged at 1000 x g for 15 min at +4 °C. Serum and skim milk were collected, inactivated at 56 °C for 90 min and stored at -20 °C until analysis.

2.3 Antibody detection

Presence of *N. caninum*-specific antibodies in bovine and canine samples were demonstrated by iscom ELISA as previously described (Björkman et al., 1994; Björkman et al., 1997). Sera and milk were diluted 1:100 and 1:2, respectively, in PBS-Tween before analysis. Positive and negative sera were heat-treated in the same manner as the samples and included in each analysis as controls. All samples and controls were analysed in duplicates and the optical density (OD) was measured at 450 nm. Mean OD values of the duplicates were calculated and subsequently correlated to a positive control serum with a mean OD value of 1.00.

Bovine sera with corrected OD >0.35 were considered positive and for canine sera OD = 0.20 was used as cut-off.

2.4 Data analysis

Pedigree data were obtained from the farm records and from the cattle identity archive issued by the Department of Livestock Development, Ministry of Agriculture and Co-operatives of Thailand. The association between the serological status in dams and their offspring was measured and compared using 2 x 2 tables and Fisher's exact test. To study the mode of *N. caninum* transmissions over the years, each dam-offspring pair was allowed to contribute to the comparison only once, i.e. only the test results from the first sampling where both the dam and the offspring were present were considered.

Apparent vertical transmission rates were calculated as the proportion of antibody-positive offspring born to antibody-positive dams and the horizontal transmission rates as the proportion of antibody-positive offspring born to antibody-negative dams. Estimates and 95% confidence intervals of these transmission rates were assessed using WinEpiscope software version 2.0 (Thrusfield et al., 2001). The seropositive and seronegative seroconversion rates were calculated as previously described by Pan et al. (2004).

Data management and statistical analysis were carried out using analysis in Stata Statistical Software release 8.0 (StataCorp., College Station, TX, USA) and Microsoft® Excel 2002 (Microsoft Corp., USA).

3. Results

The overall percentage of antibody-positive cattle varied between 10 and 13% (Table 1), but the variation between herds was substantial. Two herds (Herd 10 and 11) had $\geq 20\%$ seropositive animals at all samplings and consistently high bulk milk OD values, whereas Herd 5 and 9 had no seropositive animal at the last two samplings (cows antibody-positive at the previous samplings had all been culled) and also low bulk milk OD values. Five herds (Herd 5, 8, 9, 10, and 11) had a decreasing trend of within-herd seroprevalence whereas the other six herds had a higher portion of test-positive individuals at the end of the study.

Of the 424 individuals that were sampled more than once, 344 (81%) and 32 (8%) were consistently test-negative and test-positive, respectively (Table 2). Of the 48 individuals that changed serological results, 20 went from being test-negative to test-positive and 16 went from test-positive to test-negative. The other 12 individuals changed serological status in both directions between repeated samplings. Of the 48 animals that changed serological results, 29 were from herds with moderate within-herd seroprevalence rates (10-19%) and 8 were from herds having high within-herd prevalence ($\geq 20\%$) and the remaining 11 were from herds with a low seroprevalence (<10%). The rates of seropositive and seronegative conversion are presented in Table 3. The seropositive conversion rates between

the years were consistent, ranging from 4.0 to 4.6%. Conversely, seronegative conversion rates varied considerably and were between 19 and 39%. The highest rate (39.4%) of seronegative conversion was observed between the year 2001 and 2002.

The serostatus of the 412 pairs of dams and their offspring are shown in Table 4. The apparent vertical transmission rate was 58% (95% CI; 44-71%) whereas the horizontal transmission rate was 5% (95% CI; 3-7%). When the dam-offspring pair was categorized according to average within-herd seroprevalence the apparent vertical and horizontal transmission rates were 78% (18/23) and 6% (2/36), respectively, in herds with high prevalence (Table 5). No vertical transmission was observed and the horizontal transmission rate was 2% (4/167) in herds with low prevalence.

A total of 180 pairs were present more than once; 126 (70%) and 14 (7%) of these pairs had both dams and their offspring consistently negative and positive, respectively. However, 35 (19%) pairs had changed their serological status of either dam or offspring, or both, at least once.

Of the 36 sampled dogs 4 had antibodies to *N. caninum*. They belonged to herd 1, 5, and 9. The seronegative dogs were from herd 3, 5, 6, 8, 10, and 11.

4. Discussion

The present investigation showed a fairly constant overall percentage of *N. caninum* antibody-positive cattle over the four years (10-13%). In addition, we found that the infection rates varied between the herds. Previous investigations performed in Thailand have shown that the prevalence differs both between studies and between regions, varying from 6% to 70% (Suteeraparp et al., 1999; Kashiwazaki et al., 2001; Chanlun et al., 2002; Kyaw et al., 2004).

Over 89% of the individual animals that were sampled more than once had consistent serological status. However, both seropositive and seronegative conversions in individual animals were observed and interpretation of these results is complicated. Several studies have reported occurrence of seropositive conversions in cattle as a result of postnatal infection, recrudescence of a chronic *N. caninum* infection or false-positive test results (see e.g. Waldner et al., 2001; Dijkstra et al., 2002; Pfeiffer et al., 2002; Pan et al., 2004; Frössling et al., 2005). The seropositive conversion rates between the years (~4%) in the current investigation was consistent with two Canadian studies (Waldner et al., 2001; Pan et al., 2004). Even higher rates of seropositive conversion (45-55%) have been reported from the Netherlands (Dijkstra et al., 2002) and Australia (Pfeiffer et al., 2002). Based on samples from Swedish *N. caninum*-infected herds, the specificity of the iscom ELISA has been estimated to be high but not perfect (Frössling et al., 2003). Some test-positive animals could thus be expected to be false-positives. However, in the present study the number of inexplicable positive results was higher than expected. One explanation for this could be that nonspecific cross-

reactivity, e.g. to other infectious agents present in Thailand but not in northern Europe, might have altered the performance of the test and given a somewhat higher portion of false positive results.

The high seronegative conversion rate (19-39%) presented here is consistent with those of some other studies (Sager et al., 2001; Waldner et al., 2001; Pan et al., 2004). In a Canadian study, 65% of 81 seropositive cows were seronegative when they were sampled again after two years (Pan et al., 2004). Although most conversions in that study took place in animals that were moderately positive at the first sampling, strongly seropositive individuals also became seronegative. In addition, a study in Thailand has recently reported that 100% (4/4) of *N. caninum*-infected cows were seronegative at calving about one year after they had tested seropositive (Kyaw et al., 2005). However, Dijkstra et al. (2003) found that only 4% of 616 seropositive cattle in 21 *Neospora*-infected herds seroconverted over a two-year period, and suggested that most of the conversions were due to false positive or negative test results, or that the individual initially tested positive because of remaining maternal antibodies. Moreover, transient false positive results of two- to three-year-old heifers that classified as antibody-negative have been reported (Hietala and Thurmond, 1999). *N. caninum* antibody levels can fluctuate during pregnancy (Stenlund et al., 1999) and may also fall below the cut-off of the test. Recently, a study has revealed an increase of *N. caninum* antibody titers in cows after consuming oocysts, which later reverted to seronegative status (Gondim et al., 2004a). It has generally been considered that *N. caninum* infection is life-long. However, it is difficult to explain how seronegative conversion can occur between subsequent samplings to such extent as in this and in other studies unless *N. caninum*-infected animal can become free of the parasite after infection. Together with previous findings (Gondim et al., 2004a; Pan et al., 2004; Kyaw et al., 2005) our results suggest that some cattle can indeed get rid of the infection.

Vertical transmission was the most frequent route of *N. caninum* infection in the two herds with consistently high within-herd prevalence. Moreover, transplacental infection over several generations was observed in the herds with moderate seroprevalence, corroborating previous observations that the infection can remain in cattle herds for generations by this transmission route (Björkman et al., 1996; Frössling et al., 2005). Horizontal transmission was also recorded although its source could not be identified and the infection rate was low. Notably only 4 of the 36 tested farm dogs had antibodies to *N. caninum* and all seropositive dogs were present in herds with a low seroprevalence. In spite of this, it cannot be excluded that dogs were the main source of horizontal infections. Studies have shown that antibody-negative dogs can still excrete oocysts and that they could be a risk factor of infection (McAllister et al., 1998). The apparent rates of vertical and horizontal transmission in the present investigation could be either underestimates or overestimates because of the characteristics of the test and the sampling schedule i.e. where progeny was not tested immediately after birth. Due to horizontal transmission, the serological status of either the dam or its offspring, or both, may have changed since the time of birth of the offspring.

In conclusion, there was a constant overall percentage of *N. caninum* antibody-positive cattle over the years, but a considerable variation of the within-herd seroprevalence between the herds was observed. In addition, both seropositive and seronegative conversions were likely to occur in the individual cattle although most animals had consistent serological status throughout the study.

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Table 1

Number of *Neospora caninum* seropositive animals (n_{pos}) and number of total animals (n_{tot}) >3 months of age and the level of *N. caninum* antibodies in bulk milk in 11 Thai dairy herds during 2001-2004. Presence of antibodies was analysed by an *N. caninum* iscom ELISA. Optical density (OD) value >0.35 was considered positive for serum samples and result for bulk milk is presented as OD value.

Herd	Classification ^a	2001		2002		2003		2004	
		$n_{\text{pos}} / n_{\text{tot}}$ (%)	Bulk milk OD ^b	$n_{\text{pos}} / n_{\text{tot}}$ (%)	Bulk milk OD	$n_{\text{pos}} / n_{\text{tot}}$ (%)	Bulk milk OD	$n_{\text{pos}} / n_{\text{tot}}$ (%)	Bulk milk OD
1	Low	2/40 (5)	0.04	2/43 (5)	0.07	3/45 (7)	0.20	4/52 (8)	0.22
2	Low	1/28 (4)	0.06	0/19 (0)	0.09	1/26 (4)	0.06	3/34 (9)	0.10
3	Moderate	4/38 (11)	0.09	5/39 (13)	0.15	7/44 (16)	0.19	5/37 (14)	0.35
4	Moderate	1/38 (3)	0.09	3/30 (10)	0.05	7/41 (17)	0.38	5/35 (14)	0.55
5	Low	1/25 (4)	0.12	0/29 (0)	0.03	0/35 (0)	0.05	0/27 (0)	0.09
6	Moderate	4/37 (11)	0.22	3/39 (8)	0.72	4/36 (11)	0.57	6/39 (15)	0.71
7	Moderate	6/43 (14)	0.22	5/51 (10)	0.02	10/58 (17)	0.03	7/60 (12)	0.07
8	Low	4/28 (14)	0.31	1/31 (3)	0.03	0/29 (0)	0.06	3/24 (13)	0.26
9	Low	2/19 (11)	0.42	1/21 (5)	0.14	0/23 (0)	0.10	0/24 (0)	0.07
10	High	13/31 (42)	0.85	9/28 (32)	0.73	11/29 (38)	0.60	9/33 (27)	0.72
11	High	8/33 (24)	0.89	8/29 (28)	0.32	7/19 (37)	0.56	4/20 (20)	0.58
Overall		46/360 (13)		37/359 (10)		50/385 (13)		46/385 (12)	

^aHerds classified according to the average of within-herd seroprevalence at all samplings. Herds with average of seroprevalence $\geq 20\%$ and 10-19% were classified as high and moderate, respectively, otherwise herd was classified as low when average of within-herd seroprevalence <10%.

^b Data from Chanlun *et al.* (2002)

Table 2
Number of antibody positive samples for *Neospora caninum* and total number of serum samples collected per individual animal

Number of samples collected per animal	Number of antibody positive samples per animal (%)				Number of animals
	0	1	2	3	
1	250 (90.6)	26 (9.4)			276
2	152 (81.7)	18 (9.7)	16 (8.6)		186
3	93 (83.8)	12 (10.8)	2 (1.8)	4 (3.6)	111
4	99 (78.0)	10 (7.9)	1 (0.8)	5 (3.9)	127

Presence of antibodies was analysed by an *N. caninum* iscom ELISA. Optical density >0.35 was considered positive

Table 3

Seroconversion rate between seropositive and seronegative individual when the animals had repeated records in the years 2001, 2002, 2003, and 2004. Presence of *N. caninum* antibodies was analysed by iscom ELISA. Optical density value >0.35 was considered positive.

Period	2001-2002	2002-2003	2003-2004
Seropositive conversion (%) (-)------> (+)	4.4 (226) ¹	4.0 (224) ¹	3.9 (230) ¹
Seronegative conversion (%) (+)------> (-)	39.4 (33) ²	19.2 (26) ²	24.3 (37) ²
Total number of sample	259	250	267

¹The number in parentheses denotes the number of seronegative animals in the first year

² The number in parentheses denotes the number of seropositive animals in the first year

Table 4

Neospora caninum serological status of dams and their offspring in 11 Thai dairy herds during 2001-2004. Presence of antibodies was analysed by iscom ELISA. Optical density value >0.35 was considered positive.

Offspring	Dam		Total
	Negative	Positive	
Negative	342	22	364
Positive	18	30	48
Total	360	52	412

Fisher's exact test $p < 0.001$.

Table 5

Neospora caninum serological status of dams and their offspring of the total 412 unique pairs and the within-herd seroprevalence in 11 dairy herds during 2001-2004. Presence of antibodies was analysed by iscom ELISA. Optical density value >0.35 was considered positive.

Within-herd seroprevalence	Offspring	Dam	
		Negative	Positive
≥20 ^a	Negative	34	5
	Positive	2	18
10-19 ^b	Negative	145	11
	Positive	12	12
<10 ^c	Negative	163	6
	Positive	4	0

^aFisher's exact test $p < 0.001$.

^bFisher's exact test $p < 0.001$.

^cFisher's exact test $p = 0.867$.

