การติดเชื้อและการวินิจฉัยการแท้งลูกที่สัมพันธ์กับ *Neospora caninum* ในโคนม

นายตัน คะยอ

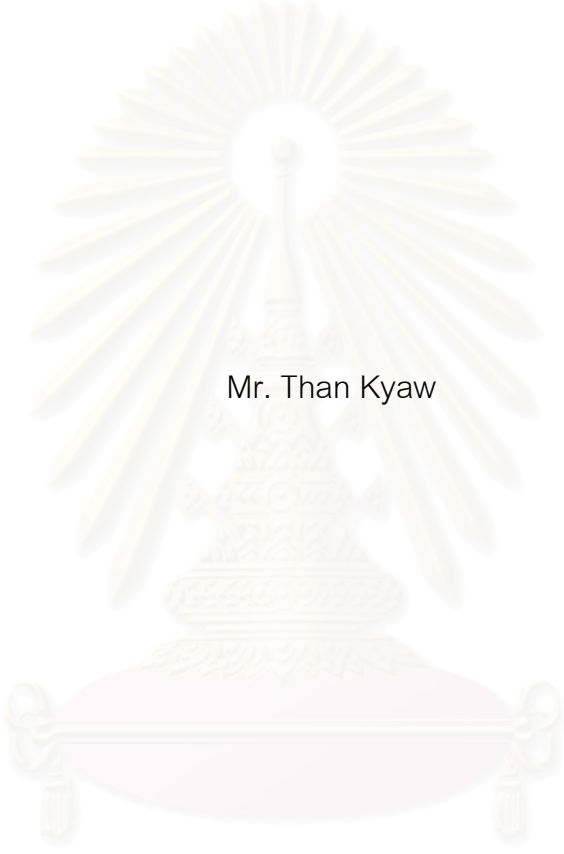# SEROPREVALENCE OF ANTIBODIES AND DIAGNOSIS OF

ABORTION RELATED TO *Neospora caninum*

IN DAIRY CATTLE

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A thesis submitted in partial fulfillment of the requirements

for the Degree of Master of Science in Theriogenology

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Faculty of Veterinary Science

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ต้น   คะยอ : การติดเชื้อและการวินิจฉัยการแท้งลูกที่สัมพันธ์กับ *Neospora caninum* ใน
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การศึกษาแบ่งเป็น 2 ส่วน ส่วนที่ 1 ได้แก่ การสำรวจความชุกของระดับภูมิคุ้มกันต่อเชื้อ
โปรโตซัว *Neospora caninum* (NC) ในฟาร์มโคนม อ.สวนป่านและสระกระเทียม จังหวัด
นครปฐม และ ส่วนที่ 2 การตรวจวินิจฉัยการแท้งลูกในโคนมสาเหตุจากเชื้อ NC ในฟาร์มโคนม 2
แห่งที่มีตรวจพบภูมิคุ้ม NC และมีประวัติการแท้งลูก

การตรวจระดับภูมิคุ้มต่อเชื้อโปรโตซัว NC โดยใช้วิธี competitive enzyme-linked
immunosorbant assay (c-ELISA) ใน 59 ฟาร์ม จำนวนแม่โค 549 ตัว พบว่า อัตราการพบ
ภูมิคุ้มกัน NC ในฝูงโคนม 20 จาก 59 ฟาร์ม (33.9%) และอัตราการพบในประชากรที่สำรวจ
30 ตัว (5.46%)   แม่โคที่มีประวัติการแท้งลูก 12 ตัว ตรวจพบมีภูมิคุ้มต่อ NC จำนวน 1 ตัว ขนาด
ของการเลี้ยงโคนมพบว่าฝูงขนาดใหญ่ (โคนมมากกว่า 21 ตัว) มีความสัมพันธ์กับจำนวนแม่โคที่
ตรวจพบภูมิคุ้มกันต่อเชื้อ โปรโตซัว NC สูงกว่าฝูงขนาดเล็ก (p=0.034) สุนัขที่เลี้ยงในฟาร์มที่
สำรวจ จำนวน 82 ตัว ตรวจพบภูมิคุ้มกันต่อเชื้อ โปรโตซัว NC 1 ตัว (1.2%)แต่สุนัขซึ่งเป็นพาหะ
ของโรคตามธรรมชาติไม่มีความพันธ์กับการตรวจพบภูมิคุ้มโรคในแม่โค

การตรวจวินิจฉัยสาเหตุการแท้งลูกจากเชื้อ NC จากฟาร์มโคนม 2 แห่ง ที่พบการแท้งลูก
ในระยะอุ้มท้อง 3-8 เดือนในจังหวัดสระบุรีและชลบุรี จำนวน 12 ตัว พบภูมิคุ้มต่อโรค NC 5 ตัว
(41.7%) ฟาร์ม 1 แห่งที่ประวัติตรวจพบภูมิคุ้มโรค NC ในแม่โคแท้ง 5/13 ตัว (38.5%) ได้ตัวอย่าง
ลูกที่แท้ง จำนวน 10 ตัวเพื่อใช้ในการศึกษาทางจุลพยาธิวิทยาและย้อมสีพิเศษด้วยวิธีอิมมูโนฮีส
โตเคมี พบว่าลูกโคแท้ง 1 ตัว อายุตั้งท้อง 4 เดือน พบเชื้อปาราสิตระยะ Tachyzoite ในเนื้อเยื่อรก

การตรวจพบการติดเชื้อ NC โดยตรวจพบระดับภูมิคุ้มต่อโรคในโคนม 5.46% และตรวจ
วินิจฉัยพบเชื้อ NC ในลูกที่แท้งเป็นรายงานแรกที่พิสูจน์ว่าเชื้อโปรโตซัวนี้เป็นสาเหตุของการแท้ง
ลูกในโคนมและควรมีการศึกษาป้องกันความสูญเสียจาก NC ในประเทศไทยต่อไป

ภาควิชา สูติศาสตร์ เธนุเวชวิทยา   ลายมือชื่อนิสิต…………………………......…………
      และวิทยาการสืบพันธุ์   ลายมือชื่ออาจารย์ที่ปรึกษา…………………....……
สาขาวิชา วิทยาการสืบพันธุ์สัตว์   ลายมือชื่ออาจารย์ที่ปรึกษาร่วม…………………......
ปีการศึกษา 2545

THAN KYAW: SEROPREVALENCE OF ANTIBODIES AND DIAGNOSIS OF ABORTION RELATED TO *Neospora caninum* IN DAIRY CATTLE

THESIS ADVISOR: ASSO. PROF. DR. PRACHIN VIRAKUL, PhD,

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The seroprevalence of antibodies to *Neospora caninum* (NC) and the relationship between seropositivity and age (heifer vs. cow), and the relationship of herd infection with herd size and with presence of dogs on the farm in dairy cattle were studied involving 549 cows and 82 dogs in 59 dairy herds in Nakhon Pathom. A competitive enzyme-linked immunosorbent assay (c-ELISA) was used to detect the NC-antibodies in the sera. Individual and herd seroprevalence of NC were 5.46% (30/549) and 33.9% (20/59) respectively. No significant associations of NC seropositivity with age of cow (heifer vs. cow; p=0.331) and of herd infection with the presence of dogs on the farm (p=0.378) were observed. The larger herd (≥ 21 cows) had significantly higher infection (p=0.034) than small herds (≤ 20 cows). Of 12 cows with abortion history, one was seropositive to NC. Seroprevalence of NC antibodies in dogs was 1.2% (1/82). This is the first NC seroprevalence study in dogs in Thailand. It was concluded that *Neospora*-infection was common in herd rather than individual level in Thailand and the presence of dogs on the farm was not always related to the herd infection and caution should be taken in the interpretation of serological tests from the farm dogs.

An immunohistochemical (IHC) examination was conducted to detect NC organisms in 12 aborted fetal tissues (3-8 months gestation) collected from 2 farms in Saraburi and Chonburi Provinces. Five of these cows (41.7%) were seropositive to NC. *Neospora*-tachyzoites were detected in the placenta of a seropositive cow aborted at 4 months of gestation. Neither the NC parasites nor tissue cysts were detected in the other fetal tissues. Also, 38.5% (5/13) of cows whose aborted fetuses were not collectable were seropositive to NC. This is the first report on the identification of NC parasite in Thailand. It was concluded that the identification of *Neospora*-parasite and high percentage of *Neospora* seropositivity in the aborting cows strongly indicated the possibility of NC as a major cause of abortions and it is important to make further studies for the control measure of this disease.

Department of Obstetrics Gynaecology and Reproduction	Student's signature ....
Field of study: Theriogenology	Advisor's signature ....
Academic year: 2002	Co-advisor's signature ....

Acknowledgements

CONTENTS

CHAPTER 1

Introduction

Neosporosis, since its first report as an unidentified protozoan in Norway in 1984 (Bjerkås *et al*., 1984), has been paid much attention and many reports have emerged on this disease being one of the major causes of abortion in cattle worldwide (Dubey and Lindsay, 1996; Dubey, 1999a, 1999b, 1999c, Anderson *et al.,* 2000; Hemphill and Gottstein, 2000; Reichel, 2000; Antony and Williamson, 2001; Dubey *et al*., 2002). Neospora parasite is prevalent in dogs and cattle (Dubey, 1999a; Wouda *et al.,* 1999b) but it is also found to be infective to other domestic animals such as sheep, goats, swine, horse and deer, including cats (Dubey and Lindsay, 1996; Dubey, 1999a). In addition to these animals there is an evidence of human infection by Neospora (Tranas *et al.,* 1999). Some of recent reports (table 1) show a widespread and significant incidence of the disease not only in cattle but also in buffaloes and dogs in different countries, including Thailand. Very few reports from the Asean countries are found in the literature.

In a recent review on neosporosis, Reichel (2000) stated that the estimated economic loss due to neosporosis was at A\$ 85 million per annum for the dairy and A\$ 25 million for beef cattle industry in Australia and NZ\$ 17.8 million for the dairy industry in New Zealand. In California dairy industry, it was estimated that about US\$ 35 million per year was lost due to abortions by neosporosis (Dubey, 1999b).

At present high incidence of neosporosis in dairy and beef cattle up to about 87% indicates the important situation of this disease in the economic aspect of cattle industry in the world. High seroprevalence in buffaloes as high as 68% (table 1) also indicates that these animals may also be a potential source of infection to cattle in Asia where both cattle and buffaloes are raised closely.

Table 1. Some recent reports on the seroprevalence of Neosporosis.

Animal	Number tested	Test Method	Positive %	Country	Source
Dairy					
	904	IFAT	6	Thailand	Suteeraparp *et al.,* 1999
	613	IFAT	44.9	Taiwan	Ooi *et al.*, 2000
	447	IFAT	14.09	Brazil	Gondim *et al.,* 1999
	266	IFAT	24	Australia	Atkinson *et al.*, 2000
	23 herds	IFAT	21.9	Quebec	Bergeron *et al.*, 2000
	780	IFAT	2	Sweden	Björkman *et al.*, 2000
	465	IFAT	26.9	USA	Dyer *et al.*, 2000
	4295	ELISA	17.1	England	Davison *et al.*, 1999a
	1924	ELISA	5.6	France	Ould-Amrouche *et al.*, 1999
	200	ELISA	5.5	Vietnam	Huong *et al.,* 1998
	50 herds	ELISA	87	The Netherlands	Wouda *et al.*, 1999a
	1003	ELISA	56	Mexico	Morales *et al.*, 2001
	266	Immunoblot	29	Australia	Atkinson *et al.*, 2000
Beef					
	2585	CI-ELISA	24	USA	Sanderson *et al.*, 2000
Buffalo					
	75	DAT	68	Egypt	Dubey *et al.*, 1998
	200	IFAT	1.5	Vietnam	Huong *et al.,* 1998
	1377	IFAT	34.6	Italy	Guarino *et al.*, 2000
Dog					
	13	IFAT	2.3	Taiwan	Ooi *et al.*, 2000
	1077	IFAT	7	US & Canada	Cheadle *et al.*, 1999
	134 (farm)	IFAT	21.6	Brazil	de Souza *et al.*, 2002
	152 (farm)	ELISA	23.6	-	Wouda *et al.*, 1999b
	344 (urban)	ELISA	5.5	-	Wouda *et al.*, 1999b
	48 (farm)	IFAT	31.3	Japan	Sawada et al., 1998
	198 (urban)	IFAT	7.1	Japan	Sawada et al., 1998
Human					
	1029	IFAT	6.7	-	Tranas *et al.*, 1999

CI-ELISA = Competitive inhibition Enzyme-linked Immunosorbent Assay

IFAT = Indirect Fluorescent Antibody Test

DAT = Direct Agglutination Test

CHAPTER 2

Literature Review

2.1 Causal agent

The causal organism of neosporosis in cattle is *Neospora caninum*, a cyst-forming protozoan or coccidia in the family *Sarcocystidae*, phylum *Apicomplexa* and it was first named by Dubey and his colleagues in 1988 (Dubey *et al.,* 1988a). Ten years later, a new serologically distinct species of Neospora, *N. hughesi,* isolated from an adult horse was reported (Marsh *et al*., 1998) but its presence and relationship to cattle and other animals has not been known yet.

2.2 Life cycle of *N. caninum*

Although the complete life cycle of Neospora parasite is not fully known, it basically comprises asexual and sexual part of life cycle. In infected dogs, tachyzoites and bradyzoites are asexually produced. Tachyzoites are ovoid, lunate or globular and measure 3 to 7 $\times$ 1 to 5 μm depending on the stage of development and can be found in cells of various tissues of the body while tissue cysts, mostly found in the central nervous system and peripheral nerves, are oval and as large as 107 μm long with a cyst wall thickness of up to 4 μm (Dubey *et al*., 1988a; Dubey and Lindsay, 1996; Speer *et al.*, 1999; Dubey *et al.,* 2002). Recently, more details of morphological, ultrastructural, serological and molecular distinctions of *N. caninum* from other coccidia have been reviewed by Dubey *et al.* (2002).

The definitive host in the Neospora life cycle is not known until 1998 when McAllister and coworkers experimentally proved dogs as definitive hosts (McAllister *et* al., 1998a). It was later confirmed by Lindsay *et al.* (1999) and Basso *et* al. (2001) were able to isolate *N. caninum* oocsts from the feces of a naturally infected dog. Dijkstra *et al.* (2001) demonstrated that dogs shed oocysts after ingestion of bovine placenta infected with *N. caninum*. In contrast to dogs, red foxes (*Vulpes vulpes*) did not shed oocysts after feeding infected intermediate host tissues (Schares *et al*., 2002).

The oocysts containing sporozoites are sexually produced and excreted by the infected dogs. These oocysts are sporulated within 3 days after shedding and ready to infect to the susceptible animals (McAllister *et al.*, 1998a; Dubey, 1999b). The dog can also be an intermediate host as well. From the existing knowledge it has been suspected that other definitive hosts, apart from dogs, such as wild canids, may exist (Fig. 1). Cats and Mustela species are not definitive hosts (McAllister *et al.*, 1998b; McAllister *et al.*, 1999).
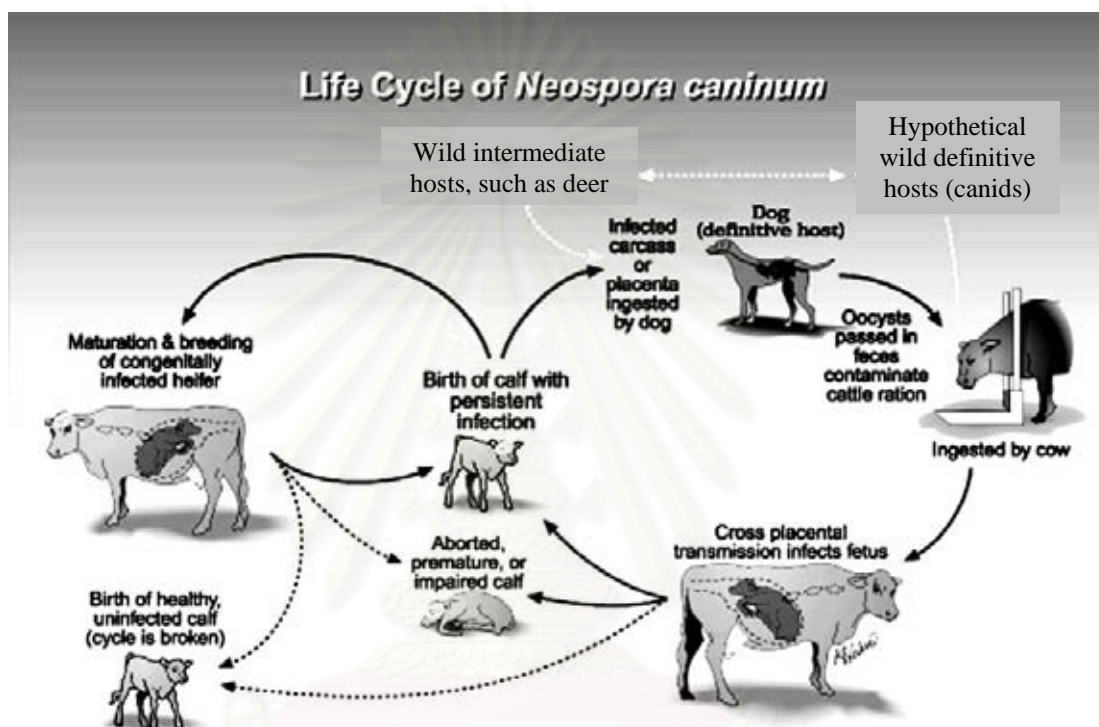
Fig. 1. Life cycle of *Neospora caninum* with a suggested sylvatic path.
(Diagram – M McAllister; slightly modified)

2.3   Disease transmission

Two types of Neospora transmission are recognized: (1) Vertical propagation or transplacental transmission in which the parasite is passed from mother to the fetus and (2) horizontal transmission in which a two-host life cycle is needed to infect the cattle by ingestion of oocysts shed by the definitive host (Anderson *et al.,* 2000; Bergeron *et al.*, 2000). In a study of dogs in dairy farms with known neosporosis prevalence, 23.6% of dogs showed seropositive while only 5.5% of urban dogs were seropositive and there was a relationship of *N. caninum* infection between dog and cattle (Wouda *et al.,* 1999b). Similarly, high prevalence of antibodies to *N. caninum* in farm dogs was also

reported (de Souza *et al.*, 2002). Buxton *et al.* (1997) reported that *N. caninum* infected wild foxes in Belgium. This indicates that the presence of dogs (or wild canids) on the cattle farms is very important in the horizontal transmission of the disease.

The other important horizontal transmission to be noted is contaminated food and water which could increase the abortion risk associated with neosporosis (McAllister *et al.*, 1998a; Anderson *et al.*, 2000). The recent finding of Dijkstra *et al.* (2002a) strongly supports the postnatal infection of the cattle from the feces of infected farm dogs which consumed infected fetal fluids or placental materials. Point source exposure of cattle to *N caninum* in specific age-group of cows occurring abortion outbreak was reported (McAllister *et al.*, 2000; Dijkstra *et al.*, 2002b).

Experiments demonstrated that calves could be infected by oral inoculation of *N. caninum* oocysts collected from the infected dog (De Marez *et al.*, 1999) and by feeding colostrum inoculated with tachyzoites (Uggla *et al.*, 1998). Although natural infection by this route has not been assessed yet, cow-to-calf transmission through infected or contaminated milk may also be an important route of horizontal transmission. Also no horizontal cow to cow transmission has not been reported yet.

The vertical transmission is a major route involved in the spread of *N. caninum* in the cattle herds and there is only a low level of horizontal transmission (Anderson *et al.*, 1997; Davison *et al.,* 1999a; Davison *et al.*, 1999b). The vertical transmission may be as high as 95.2% in a total of 124 seropositive dams and calves (Davison *et al.*, 1999b). Similar result has been reported in Quebec with 44.4% of seropositivity by vertical transmission (Bergeron *et al.*, 2000). Hietala and Thurmond (1999) suggested that vertical transmission of *N. caninum* occurred mostly in late gestation period rather than postnatal infection and dams remained seropositive throughout their life.

Although early embryonic death may occur due to *N. caninum* by vertical transmission, no reports are available at present. It was experimentally proved that zona pellucida of pre-implantation stage embryo could protect invasion of *N. caninum* (Bielanski *et al.*,

2002). It is assumed that the *Neospora* transmission through semen is improbable. But in embryo transfer, infection to the fetuses conceived by the seropositive dams were high while no infection was found in fetuses of seronegative dams (Baillargeon *et al.*, 2001). Therefore, on farms where embryo transfer is practised should use seronegative recipients.

2.4   Clinical symptoms

Cows: The *N. caninum* infected cows show no prominent clinical signs. The most dramatic and visible effect of neosporosis is abortion and the aborted cows show no clinical illness (Dubey, 1999a; Reichel, 2000; Anderson *et al.,* 2000). Abortion may occur at any stage of gestation but usually occurs during mid gestation (4th to 6th month of gestation). Aborted fetuses are usually autolysed with no gross lesions and placentas are not retained. Fetuses may die *in utero*, be resorbed, mummified, stillborn, born alive but diseased, or born clinically normal but chronically infected (Dubey, 1999a). Lesions in aborted fetuses are usually only visible upon histological examination and are located mainly in the brain although pericarditis, myocarditis, hepatitis, pneumonia and nephritis may also be seen (Dubey and Lindsay, 1996; Wouda *et al.*, 1997; Anderson *et al.*, 2000). Abortion occurs in the epidemic (abortion storm) rather than in the endemic (sporadic) form in herds at the rate of 5 to 33% (Wouda *et al.*, 1997; Reichel, 2000).

Abortion storm is defined as a cluster of abortions within a 4-week period involving more than 15 % of the animals (pregnant cows and heifers) at risk (Moen *et al.*, 1998). Cows aborting during the outbreaks and *N. caninum* seropositive non-aborting cows had a 2- to 3-fold increased risk of abortion compared with *N. caninum* seronegative cows (Paré *et al.*, 1997; Moen *et al.*, 1998). In the study of seropositive descendents, Wouda *et al.* (1998) showed that seropositive cows had a 3-fold increased abortion risk (26.5%) compared with seronegative F_1 cows (8.4%). Repeat abortions in *Neospora*-infected cows are assumed to be due to the recrudescence of the parasite rather than the result of a recent reinfection (Barr *et al.*, 1993; Wouda *et al.*, 1999b). It is not known what triggers the parasites to reactivate.

Calves: In cattle clinical signs can only be observed in calves less than 2 months (Dubey, 1999b). Congenitally infected calves born alive may be underweight, unable to rise with limbs flexed or hyper-extended or signs of ataxia (Dubey, 1999a). When calves, 6 months of age, were experimentally infected by feeding colostrum inoculated with tachyzoites, they showed transient fever and blood-stained diarrhea 1-2 weeks after inoculation (Uggla *et al.*, 1998).

Dogs: In dogs these neuromuscular signs, especially hind limb paresis or rigid hyperextension of the limbs, can be observed not only in pups (Dubey and Lindsay, 1996) but also in adult dogs (Lindsay and Dubey, 2000; Lorenzo *et al.*, 2002). In the later case the dog showed progressive pelvic weakness and difficulty in jumping. Other dysfunctions are difficulty in swallowing, paralysis of jaw, muscle flaccidity and muscle atrophy (Lindsay and Dubey, 2000).

2.5   Effect on milk production

Although no clinical signs, except abortion, are observable in neospora infected cows, it is possible that several organs such as brain, liver, heart and kidneys, will be affected for their normal physiological functions in these cows. Consequently, this will lead to a reduced production of cows. Thurmond and Hietala (1997) first reported the reduced production of milk in first-lactation dairy cows infected with Neospora. They found that milk and fat production of seropositive cows was (1.14 kg/cow/d and 0.064 kg/cow/d, respectively) less than seronegative cows. The study of Hernandez *et al.* (2001) for more than 4 successive lactations showed that seropositive cows produced 1.27 kg/cow/d less than seronegative cows. After adjustment for the effect of lactation, season, mastitis and lameness, the decrease in milk production was 1.14 kg/cow/d. This led to a loss of US$128/cow based on the 305 day mature equivalent production. In contrast to these reports, Moen and Wouda (1995) found that the lactating cows often increased milk production up to two liters per day after abortion. This may be because the aborted cows, as mentioned, are clinically not sick and the organisms may not affect the physiological functions of these cows.

2.6   Effect on weight gain in beef calves

A very few papers have been reported on the effect of Neospora in beef cattle performance. In seropositive beef steers, a significant reduction of average daily gain (0.05 to 0.17 kg/d), impaired feed efficiency and reduction of carcass weight were reported with an estimated loss of US$15.62/calf during post weaning period (Barling *et al*., 2000; Barling *et al*., 2001).

2.7   Zoonotic potential of *N. caninum*

The fetuses of monkeys were experimentally infected with *N. caninum* since 1994 (Barr *et al,* 1994)*.* It is possible that the people (e.g., veterinarians and farmers) working with cattle infected with *Neospora* can also be infected with the parasites. Possibly, the first report concerning *anti-Neospora caninum* antibody detection in human sera was published in Korea in 1998 (Nam *et al*., 1998). These authors tested both *Toxoplasma gondii* (*T. gondii*) positive and negative sera by ELISA, western blot and IFA (6.7% of *T. gondii* positive sera cross-reacted *N. caninum* antigen and 0.9% *Toxoplasma* negative sera reacted with *Neospora antigen* by ELISA). They suggested the possibility of human infection with *N. caninum*, although the positive rate was very low. In a study of 76 women with a history of repeated abortion or intrauterine death of fetuses in Denmark, no antibodies to the *Neospora* parasites were detected by ELISA (Petersen *et al.,* 1999). Similarly, no seropositivity to the parasite in 247 human sera of blood donors and agricultural workers was reported in Ireland (Graham *et al*., 1999). According to these reports it seems that human cannot be infected or may resist to the Neospora protozoan. But the study of Tranas *et al.* (1999) indicates the zoonotic potential of the parasites. These authors studied 1,029 serum samples from California where major cause of abortions in dairy cattle was recognized due to *N. caninum*. They found that 6.7% seropositivity to the parasite by IFA. Seventy-two percent of these were seronegative to *T. gondii*. Further study is necessary to find out the extend and importance in human infection.

2.8   Diagnosis

The prior importance for the diagnosis of neosporosis may be by tracing the records and history. There is highly significant association between seropositivity and history of abortion (Atkinson *et al*., 2000). As there are no distinct clinical signs of neosporosis in cattle except abortion, abortion only may not help very much in the diagnosis. Mostly the help of diagnostic laboratories may facilitate in identifying neosporosis.

Immunohistopathology: For a definitive and confirmative diagnosis of neospora infection, histological and immunohistochemical tests are required (Dubey, 1999b). The samples of aborted fetuses (brain, heart and liver), placentas, fetal fluid and maternal sera are required for the test. But in most cases the aborted fetuses are autolysed and not suitable for the normal routine diagnostic procedures. Fetal fluid and presuckling serum may be indicative of congenital neosporosis but a lack of *N. caninum* antibody does not exclude neosporosis because there might not be enough time for the fetus to synthesize antibodies or the fetus might not have been immunologically competent (Dubey, 1995). Since only a few viable *N. caninum* organisms can be found in the autolysed tissues and not visible with H&E stained sections, the use of immunohistochemical techniques is required for a definitive diagnosis (Dubey, 1999b). The fetal brain tissue is most commonly used for the diagnosis and it usually consists of necrosis and non-suppurative focal inflammation (Dubey, 1999a). Wouda *et al.* (1997) reported that tachyzoites were identified immunohistochemically in 85% of the brains, 14% of the hearts, and 26% of the livers in confirmed neospora infected fetuses; tissue cysts were found only in the brain.

Despite morphological similarities between *N. caninum* and *T. gondii*, there are some features to distinguish between these two species. To some extend, these will reduce the confusion caused in the diagnosis (table 2) but it may need complex instruments like electron-microscope. Recently, Dubey *et* al. (2002) reviewed and described more detailed differences between *N. caninum* and other related coccidia.

Table 2. Principle distinguishing morphological features between *N. caninum* and
T. gondii (Dubey e*t al.*, 1988a; Jones *et al.*, 1997).

	Features	*T. gondii*	*N. caninum*
1	Tachyzoites	Lie in parasitophorous vacuole	Lie in host cell cytoplasm without a parasitophorous vacuole*
2	Rhoptries	Few (4-6)	Numerous (more than 11)
3	Cyst wall	Thin (0.5μm)	Thick (1-4μm)
4	Cyst	Can present in many body cells	Identified only in CNS

* Parasitophorous vacuoles were found in cell culture (Dubey *et al.*, 1988b) and later in the host cell cytoplasm (Dubey *et al.*, 2002)

Serology: The most ensuring method of diagnosing the disease is serological identification. Various methods of serological tests have been used for the detection of neosporosis in cattle. These include direct agglutination test (DAT), immunoblot analysis, IFAT (Indirect Fluorescent Antibody Test), ELISA (Enzyme Linked Immunosorbent Assay), and PCR (Polymerase chain reaction) (see table 1). Some modified tests have also been used for the improvement of sensitivity and specificity of *N. caninum*; e.g., the use of immune stimulating complex ELISA (iscom ELISA) (Björkman and Lunden, 1998; Slotved *et al.*, 1999), monoclonal antibody based competitive inhibition ELISA (CI-ELISA) (Baszler *et al.*, 1996; Baszler *et al.*, 2001), quantitative competitive polymerase chain reaction (QC-PCR) (Sanderson *et al.*, 2000), and IgG avidity ELISA (Björkman *et al.*, 1999). The use of iscom ELISA is also recommended for screening specific antibodies against *N. caninum* in the fetal fluid (Slotved *et al.*, 1999).

Antibodies against *N. caninum* could be detected not only in the sera and milk (Björkman and Lunden, 1998; Ooi *et al.*, 2000; Chanlun, 2002) but also in the vaginal secretions and in the saliva of cattle (Ooi *et al.*, 2000). The later report is the first demonstration of the presence of antibodies in the vaginal secretions and saliva of cattle. Antibody titers of infected cows are higher within 2 months of calving than other times (Atkinson *et al.*, 2000).

Björkman *et al.* (1999) showed the possibility of differentiating recent and chronic neosporosis in experimentally infected calves by using IgG avidity ELISA analysis. Three weeks after infection the IgG avidity was 9-18% and 24 weeks later it had increased to 58-76%. In cattle naturally infected for more than 6 months, all had an avidity value of greater than 50%. Maley *et al.* (2001), after 1-year study in calves, agreed that IgG avidity ELISA was useful in distinguishing between recent and chronic infection. This finding will be of value in epidemiological studies in neospora infection in cattle. One common problem of diagnosis using serological tests is to assess a definitive cut-off titer. This is because the titer and absorbance values depend on the antigen composition, secondary antibodies, age of animals and other reagents (Dubey *et* al., 1999a).

ELISA or IFAT test are useful for the diagnosis of neosporosis as there is no significant cross-reactivity with the most related protozoan *T. gondii* (Dubey *et al.*, 1999a; Osawa *et al.*, 1998). Although most of the advanced techniques are highly specific and sensitive in detecting *N. caninum* infection, limitations are their expensiveness, time consuming and need of skills.

Romand *et al.* (1998) used direct agglutination test to detect neospora antibodies in the sera and suggested the reliability of the method in the diagnostic purpose. Packham *et al.* (1998) reported that modified direct agglutination test (MAT) was more sensitive (100%) and specific (97%) than ELISA or IFAT for both naturally and experimentally infected animals. They suggested that the method was easy to use on larger samples and useful in testing sera of any species without requiring special equipment. Therefore, this test may become the choice for the diagnostic purpose. Although the agglutination tests are cheaper and easier, no extensive use of the technique has not been found yet.

2.9   Control and prevention

Although there were successful treatments for neosporosis in dogs with clindamycin, sulphonamides and/or pyrimethamine (Barber and Trees, 1996), no report has been found for the treatment in the livestock animals. Experimentally, intracelluar multiplication

of *N. caninum* tachyzoites can be effectively inhibited by artemisinin, an anticoccidiosis drug, in cell cultures without toxicity to the host cells (Kim *et al*., 2002).

With respect to preventive purpose vaccines are still at their early stage of research (Andrianarivo *et al*., 1999). Lack of effective drugs for the treatment and commercially available effective vaccines for prevention of neospora infection becomes the most important problem in the control and preventive work of the disease until now. Recently, the Intervet company has introduced a killed neospora vaccine but the efficacy of these vaccines is still to be verified. The danger and spread of the disease may be even more problematic in countries where the disease occurrence has not been identified yet. Since the dog has been proved as definitive host of *N. caninum* life cycle (McAllister *et al*., 1998a; Lindsay *et al*., 1999), farm dogs become the important factor for controlling neospora abortion in dairy herds. Briefly, a considerable measures of preventive and control methods can be achieved mainly by:

1. selective culling of infected or seropositive animals in the herd and preventing the risk of introducing infected replacement cattle,

2. careful removal of aborted fetuses and associated materials as placentas and fetal membranes to protect eating by dogs and other intermediate hosts, and

3. serotesting farm dogs and removal of positive ones from the farm and taking care of the possible fecal contamination of feed and water by infected dogs.

2.10   Neosporosis in Thailand

Thailand has a large cattle population of about 5.5 million (343,680 dairy and 5,128,600 beef) and over 1.7 million buffaloes (DLD, 2001). These animals involve in the important economic sector of the country through milk and meat production and agricultural works. Neosporosis is an important disease which affect cattle production by abortions, repeat abortions, stillbirth and birth of weak calves.

Concerning with the neosporosis in cattle industry in Thailand, only two published reports, until now, are available (Suteeraparp *et al*., 1999; Kashiwazaki, 2001). The seroprevalence of the parasite in the twelve provinces of central Thailand was found to be 6% of 904 tested cattle (Suteeraparp *et al*., 1999). The seroprevalence in different provinces ranged from 0 to 12.5% (the highest in Ratchburi). Apart from these two reports, Chanlun (2002), in his Master's degree thesis, demonstrated detection of antibodies to Neospora in bulk milk and showed the common occurrence of neosporosis among dairy herds in the Northeast Thailand. Also there is no confirmed record of Neospora infected dairy farms causing abortions in Thailand. All these show the potential of disease spread in Thailand. Very few reports on this disease in Thailand indicate the need of more exploration in the extent of the disease in the cattle industry.

2.11   Conclusion

The present study was intended to partly fulfill this need and expected to yield more information regarding the spread and involvement of the disease in some dairy herds in Nakhon Pathom, Chonburi and Saraburi provinces.

2.12   Objectives of the study

- To study the seroprevalence of *N. caninum* antibodies in dairy herds in Sakatium and Soanban subdistricts of Nakhon Pathom Province

- To study whether seroprevalence is related to the age of cow (heifer vs. cow), and relationship of herd infection with herd size and with the presence of dogs on the farm

- To attempt to identify neospora parasite in the aborted fetal tissues

CHAPTER 3

Materials and methods

This study included two parts: (1) seroprevalence study using cross-sectional survey design in two sub-districts in Nakhon Pathom Province and (2) immunohistochemical study of aborted fetuses from two farms of other Provinces (Chonburi and Saraburi).

3.1   Seroprevalence study

3.1.1   Target Population and Sample size

The population targeted was 5262 dairy cattle from 308 herds in two districts, Sakatium and Soanban, in Nakhon Pathom Province. These areas were chosen for the study because the previous report (Suteeraparp *et al.,* 1999) showed the seroprevalence of antibodies to *N. caninum* as 6.9% (4/58) but it was not clear whether this finding was representative to depict the extend of disease exposure in the herd level in this region. Herd size in these areas ranged from 3 to 50 animals. The number of cattle to be sampled from these herds were calculated using the following formula (Johnson, 1984):

$$n = \frac{\left[z^2_{(\alpha/2)} \right] p.q}{\left(E^2 \right)}$$

where: p = proportion of observed value, q = 1- p, E = level of estimated error
           rate, and

     Z = Value from Z - table at α/2 level.

Assuming 0.06 as the proportion of seroprevalence in dairy cattle (Suteeraparp *et al.,* 1999) and setting error rate at 0.02 and 95% confidence level, the estimated sample size was 541(=549).

For choosing the sample size to estimate the presence of disease in a group of animals (a herd) the following formula (Thrusfield, 1986) was used:

$$n = [1 - (1 - \alpha)^{1/d}][N - \frac{d}{2}] + 1$$

    where: N = the herd size

d = number of diseased animals in the population

α = desired confidence level (at least one case of disease in the sample)

With a herd size of 50 and assuming the number of infected animals in the population as 0.25 with 95% confidence level, the sample size required from each herd for detecting at least one case was 10 animals.

3.1.2 Sampling

Because the herd size was within the range of 3 to 50 animals, two herd sizes, small (3-20) and medium (21-50), were categorized and approximately equal number of animals from each category were randomly selected for the blood sample collection.

3.1.3 Blood sample (sera) and data collection

Five hundred and forty-nine blood samples were collected from randomly selected animals from 59 randomly selected herds. Blood samples were obtained from the coccygeal or jugular veins. Blood samples were centrifuged at 2500g for 5 minutes and sera were stored at -20°C until serological tests were done. For the farms in which the number of animals were 10 or below, blood samples were collected from all animals. Herd size, the number of heifers (1 to 2 years) and cows (3 years and above), abortion history of cows, and age and number of dogs present on each farm were recorded. Blood samples of 82 dogs were also collected to check the antibody status and to relate the risk of the presence of dogs in the farm. The cephalic or saphenous veins were used in the blood sample collection. Sera were stored at -20°C until tested.

3.1.4 Serological test

Brucella abortus: On the day of blood collection all cattle sera were tested for Brucellosis by rapid plate agglutination test (40 μl serum + 30 μl antigen). The results were read after 5 minutes. The sera which reacted were assumed to be seropositive to *B. abortus*.

N. caninum: The sera were detected for the antibodies to *N. caninum* using competitive enzyme-linked immunosorbent assay (c-ELISA) technique with commercially available test kits (VMRD, Inc., USA). Sera, reagents and 96 well *N. caninum* antigen-coated microplates were brought to room temperature before testing. Eighty microliter of each sample sera, positive control (2 wells), and negative control (3 wells) sera were placed in the wells of transfer plates according to the prepared setup records. Using a multichannel micropipette set at 50 μl, the samples were transferred to the *N. caninum* antigen-coated microplates and incubated for 1 hour at room temperature (21-25°C). The plates were gently mixed by shaking while incubating. Then the wells were emptied. The remaining sera and controls were removed by striking the inverted wells 4 times on a clean paper towel. Each well was washed 4 times with 200 μl of wash solution by using multichannel pipette. After every washes the plates were struck 4 times on the paper towel to remove residual wash solution. Fifty microliter of diluted, horseradish peroxidase-labelled *N. caninum*-specific monoclonal antibody was added to each well and the plates were incubated for 20 minutes at room temperature. Then the plates were washed 4 times as mentioned above. To each well 50 μl of substrate solution was added, mixed and incubated for 20 minutes at room temperature covering the plates with aluminium foils. After incubation, 50 μl of stop solution was added to each well and gently mixed by tapping the side of the plates several times. Immediately after adding the stop solution the plates were read on a microplate reader (Titertek Multiskan Plus, Finland) set at a wave length of 650 nm optical density (OD). The percent inhibition of antibodies to the antigens were calculated by using the formula:

% Inhibition = (100 — [(sample OD x 100) / (mean negative control OD)]

The samples with the values of $\geq$ 30% inhibition were regarded as positive and those with the values of < 30% inhibition were regarded as negative.

The dog sera were also tested and OD values were calculated similarly.

3.2 Immunohistochemical and histopathological studies of aborted fetal tissues

3.2.1 Farm history and sample collection

Two farms (Somboon farm in Chonburi and Watcharin farm in Saraburi Provinces) were chosen for this study because they have had frequent abortion cases with no clinical illness. These farms have yearly vaccination history for bovine viral diarrhoea virus disease (BVD), infectious bovine rhinotracheitis (IBR), bovine parainfluenza (PI3), and bovine respiratory syncytial virus disease (BRSV) for 5 years. Brucellosis is also negative in these farms. Therefore Neospora infection was suspected as a causal agent for the abortions. Blood and fetal tissue samples of aborted cows from these farms were collected during the period of November 2001 to September 2002. Ten percent neutral formalin was used as preservative at the time of fetal tissue collection. Blood samples of dams were also collected and sera were separated, stored and tested as before.

3.2.2 Immunohistochemical method (IHC)

The fetal tissues fixed in 10% neutral buffered formalin were paraffin embedded and the tissues were cut at a thickness of 4-5 μm using a microtome (Leica Rotary, Germany). For positive control, a goat heart loaded with *N. caninum* tachyzoites (supplied by McAllister, Illinois, USA) was used. The tissue sections, including positive control, were floated in the warm water (45-50°C, 2 min), mounted on the slides (precoated with 2% 3-aminopropylinetriethoxysilane in acetone) and dried in an oven (60°C) for 45 min. The slides were then deparaffinized in xylene (3 changes, 5 min each), 2 min in xylene+absolute alcohol and 2 min each in graduated alcohols (100%, 95%, 80%, and 70%). The slides were placed in running water, distilled water (DW) and PBS (phosphate buffered saline, Appendix A) solution for 5 min each. For blocking endogenous peroxidase, the slides were treated with 3% H_2O_2 in methanol for 30 min at room temperature. The slides were washed in DW for 5 min and in PBS 2 times for 5 min each. For the antigen retrieval, the slides were placed in a container of 0.01 M citrate buffer (Appendix B), pH 6 and heated in the microwave (1200 W) set at high power for 5 min. After removing the slides from the microwave and cooling for 30 min, they were rinsed in PBS 3 times for 5 min each. To block nonspecific binding, the slides were applied with

10% BSA (bovine serum albumin, Fluka, Switzerland) for 30 min in a humid chamber at 37°C. The slides were then rinsed 3 times in PBS for 5 min each. The primary anti-*N. caninum* rabbit serum diluted at 1:10,000 with PBS was applied to the slides and kept overnight (about 15 hours) at 4°C. (Primary anti-rabbit serum produced by inoculating rabbit 9L with NC-beef tachyzoites was supplied by McAllister, Illinois, USA). The slides were rinsed in PBS 3 times for 5 min each and applied with biotinylated secondary antibody (goat anti-rabbit IgG, Dako, Denmark) at 1:400 dilution with PBS and incubated at 37°C for 30 min in humid chamber. After rinsing the slides with PBS 3 times for 5 min each, they were applied with avidin-biotin complex (ABC, Dako, Denmark), which was prepared 30 min before the application. The slides were then incubated at 37°C for 30 min in a humid chamber and rinsed with PBS 3 times for 5 min each. The slides were put into the DAB substrate (3,3'-diamino-benzidine, Sigma, USA) container for 8 min and washed in running water for 5 min. Mayer's hematoxylin was applied to the slides for counterstaining and they were washed in running water for 5 min. The slides were dehydrated in 95% ethanol, absolute ethanol (2 steps), xylene+absolute ethanol, xyline (2 steps), for 2 min each. Finally the slides were mounted with DPX (BDH, England). Negative control slide was treated similarly except for the step of which the application of primary antibody was substituted by PBS. (A flow chart of IHC staining is added in the Appendix C).

3.2.3 Hematoxylin and Eosin (H&E) staining

Formalin-fixed, paraffin-embedded fetal tissues were cut as mentioned in the immunohistochemical method. The sections were deparaffinized in xylene (2 steps, 10 min each), hydrated in the absolute alcohol (2 steps, 2 min each), 90% and 70% alcohol (2 min each). After washing the slides in the running water (5 min), the slides were dipped in Mayer's Hematoxylin for 6 min and washed again. The slides were dipped once in acid alcohol (10% glacial acetic acid in 95% alcohol) to prevent staining of adhesives used to attach sections on the slides and washed in running water (5 min). Then the slides were dipped (4 times) in the saturated aqueous lithium carbonate for recolouring of the nuclei and washed with running water (5 min). The slides were counterstained with eosin for 45 seconds. Dehydration was done dipping the slides in

95% alcohol (3 dips), in absolute alcohol (2 steps, 2 min each), and in xyline (2 steps, 2 min each). Finally the slides were mounted with DPX.

3.3   Statistical analysis

Chi-square test was used for analysing the relationship between seropositivity and age group (heifer vs. cow), relationship between herd infection and herd size and between herd infection and the presence of dogs on the farm. The tests for block effect of each of two herd sizes and presence or absence of dogs on the farm were made separately to check their influences on the herd infection. Fisher's exact test was used where the number of observations were less than 5.

CHAPTER 4

Results

4.1   Seroprevalence study

4.1.1 Cows

The results of seroprevalence of antibodies to *N. caninum* and *B. abortus* in cows and herds are shown in table 3. Of 549 cows tested, 30 (5.46%) were found to be seropositive to *N. caninum*. Seropositivity to *B. abortus* was also found in 14 cows (2.55%). Two of *Neospora*-seropositive cows were also seropositive to *B. abortus*. The % inhibition value of the sera of *Neospora*-seropositive cows ranged from 30.7% to 94.6% with an average value of 70.2% $\pm$ 24.8 SD. Out of 12 cows with abortion history, one cow was seropositive to *N. caninum* with an inhibition value of 88.92% but they all were seronegative to *B. abortus* (table 4).

Table 3. Cow and herd seroprevalence of antibodies to *N. caninum and B. abortus*.

	N. caninum		*B. abortus*	*Both*
	+ ve	% inhibition (Mean $\pm$ SD)	+ ve	+ ve
Cows	5.46% (30/549)	70.2 $\pm$ 24.8	2.55% (14/549)	0.36% (2/549)
Herds	33.9% (20/59)	-	16.95% (10/59)	8.47% (5/59)

Table 4. Serology of cows with abortion history.

Number of cows	*N. caninum*		*B. abortus*	
	+ ve	- ve	+ ve	- ve
12	1	11	0	12

When all seropositive cows were categorized into 3 groups in accordance with the % inhibition values (low, 30 to 50%; medium, 51 to 70%; and high, 71% and above), most of the cows (60%) had a high % inhibition values (Fig. 2).
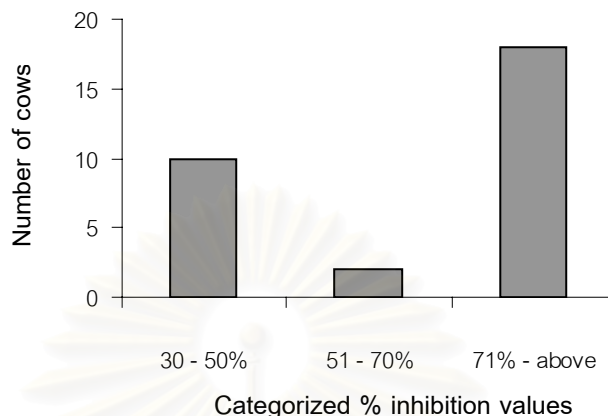
Fig. 2. Distribution of seropositive cows grouped by categorized % inhibition values.

4.1.2 Herds

Herd seroprevalence to *N. caninum* was found to be 33.9% (20/59) (table 3). Three (15%, 3/20) of these seropositive herds had no dogs.

4.1.3 Dogs

Forty-four out of 59 farms (74.5%) had dogs. Of 89 dogs from these farms, blood of 7 dogs were not collected because 6 of them were uncatchable and one was not allowed to access to the farm. Only one dog (1.22%, 1/82), a 5 year-old male belonged to a seropositive farm, showed seropositivity to *Neospora* having 82.2% inhibition.

4.1.4 Relationship of *N. caninum* seropositivity with cow age (heifer vs. cow) and relationship of herd infection with herd size and with presence of dogs on the farm

Table 5 shows the relationship between seropositivity and age (heifer vs. cow), relationship between herd infection and herd size and between herd infection and presence or absence of dogs on the farm. The number of seropositive cows was significantly higher in large farms ($\geq$ 21 cows) than smaller ones ($\leq$ 20 cows; p=0.034) and their relationship was graphically shown in Fig. 3. Large farm were likely to have 3

times more infection than smaller farms. No significant differences between the seropositivity of heifers and cows (p=0.331) and between the presence or absence of dogs on the farm (p=0.378) were observed.

Table 5. Associations between *Neospora* seropositivity and age (heifer vs. cow), between herd infection and herd size and between herd infection and presence of dogs on the farm.

	Chi-square	p value	Odds ratio	CI (95%)
Heifer vs. cow	0.09	0.331	1.57	0.63 — 3.92
Herd size	4.51	0.034	3.32	1.07 — 10.2
Presence of dogs	0.78	0.378	1.78	0.49 — 6.46
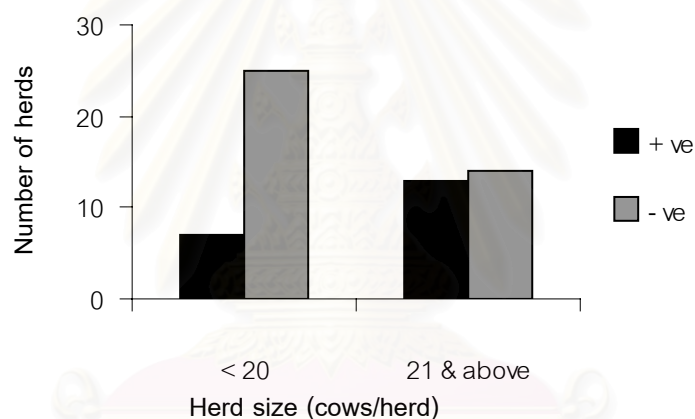
Fig. 3. Number of herds and herd size in relation to neospora seropositivity.

4.2   Immunohistochemical study of aborted tissues

Two aborted fetuses from Watcharin farm and 10 fetuses from Somboon farm and the blood samples of the dams were collected. The tissue samples collected from each aborted fetuses were listed in table 6. The blood of the other 13 cows from Somboon farm whose aborted fetuses were uncollectable were also taken for the serological tests of antibodies to *N. caninum* (table 7). Two cows of Somboon farm aborted twice during sample collection period (II NP 339, table 6 and 209, table 7). Of these two, the fetal tissues of only one cow were collectable at the second abortion (II NP 339, table 6).

Table 6. Aborted fetal tissues collected, cow serology & IHC results of tissue samples.

Sr No	Fetus ID	Farm	Gestation (mth)	Tissues collected	Neospora antibody (Dam sera)		IHC +/-
					+/-	% inhibition	
1	1D690L	Watcharin	6	Heart, kidney, lung	+	84.36	-
2	1D791A	Watcharin	8	Placenta	-	0.08	-
3	2D170D	Somboon	8	Placenta	-	5.5	-
4	2D171D	Somboon	6	Placenta	+	91.61	-
6	II NP 198	Somboon	7	Placenta	-	-5.53	-
5	II NP 199	Somboon	4	Placenta	+	91.87	+
7	II NP 197	Somboon	6	Brain, heart, placenta,	+	92.39	-
8	II NP 221	Somboon	7	Heart, kidney, placenta	-	11.37	-
9	II NP 222	Somboon	3	Heart, placenta	-	-0.42	-
10	II NP 293	Somboon	5	Brain, heart	-	9.07	-
11	II NP 294	Somboon	-	Placenta, kidney	-	9.49	-
12	IINP 339*	Somboon	6	Brain, heart, liver, lung, kidney, placenta, cotyledon	+	63.4	-

*repeat abortion (previous % inhibition value = 85.5%)

Table 7. Serology of cows whose fetuses were uncollected.

Sr No	Cow ID	Gestation (mth)	Neospora antibody (Dam sera)	
			+/-	% inhibition
1	2081	6	+	88.43
2	43008	6	+	94.89
3	4039	-	+	74.86
4	251	4	+	67.16
5	209*	4	+	92.01
6	74	8	-	4.07
7	2106	-	-	7.82
8	724	-	-	5.53
9	828	-	-	-13.14
10	473	8	-	8.55
11	42019	8	-	-13.66
12	024	8	-	6.99
13	1048	8	-	2.73

* repeat abortion (previous % inhibition value = 89.5%)

Serological tests showed that 41.7% (5/12) of aborted cows whose fetal tissues were collected, including one cow having repeat abortion (table 6) and 38.5% (5/13) of cows (sera only, table 7) were seropositive to *N. caninum*. The range of gestation age of aborted fetuses from seropositive cows was from 4 to 6 months. The % inhibition of all seropositive cows ranged from 63.4% to 94.89%. Serological tests for other diseases were not made. Of fetal tissues collected, the placenta of one seropositive cow (fetus ID, II 2NP 199, table 6) was IHC seropositive and the cow had a high % inhibition value of 91.87%. Unfortunately, we were unable to collect the fetus of this IHC positive case.

Histopathologically, the characteristic lesions of Neospora infection such as nonsuppurative necrotic foci were not found in 10 of 12 aborted fetal tissues of seropositive dams (brain, heart, liver, kidney, lung) and placentas examined. No inflammatory responses were observed in the heart, liver and kidney. The kidney of one fetus (ID 690L) from a seropositive cow was severely autolysed and lesions could not be observed. Although inflammatory reactions were not found, postmortem bacterial contaminations were observed in some loci of the lungs. Most of the placental tissues had necro-suppurative reactions and bacterial contaminations were also found.

The IHC positive placenta (fetus ID, 2NP199) is shown in figures 4, 5, 6 and 7. Some tachyzoites were found in small clusters (arrows, Fig. 4 and 5), while some were found as a scattered and single tachyzoite (Fig. 6 and 7) in the placental sections. For comparisons, tachyzoites of positive control tissue are shown in Fig. 8 and 9. Tachyzoites in the sample tissue stained well as in the positive control and there was no non-specific background staining. Most of tachyzoites in the placental tissue section were not as clearly demarcated as in the positive control tissue. This may be due to the autolysis or degradation of the organisms.

H&E stain of this placenta showed non-suppurative mononuclear infiltrations but they were not focal in nature (Fig. 10 and 11). No tachyzoites of *N. caninum* were identifiable.
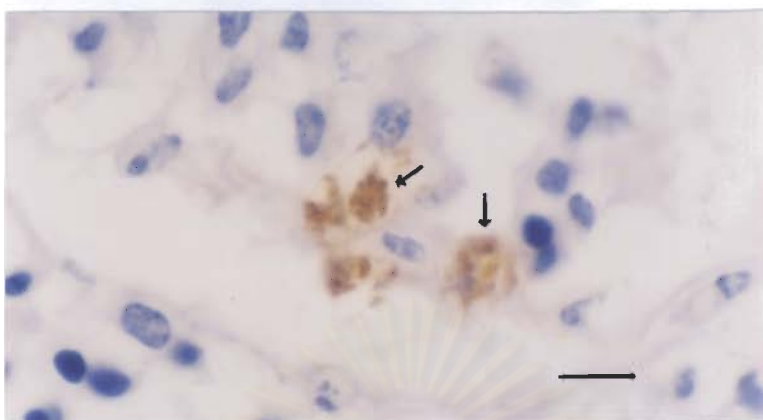
Fig. 4. Clusters of *N. caninum* tachyzoites (arrows) found in the placenta. ABC method, hematoxylin counter stain. (bar = 10 μm)
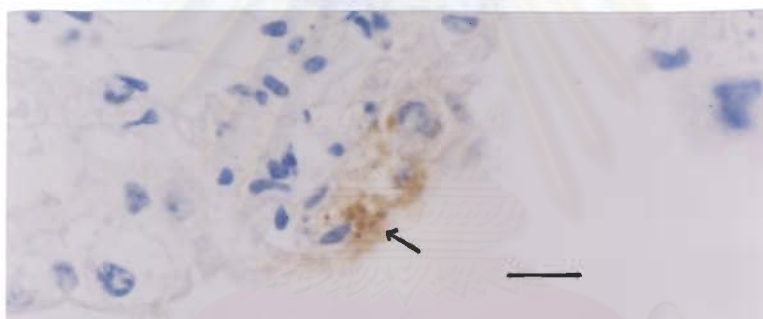
Fig. 5. A group of tachyzoites of *N. caninum* at another location of the same placenta. ABC method, hematoxylin counter stain. (bar = 12.5 μm)
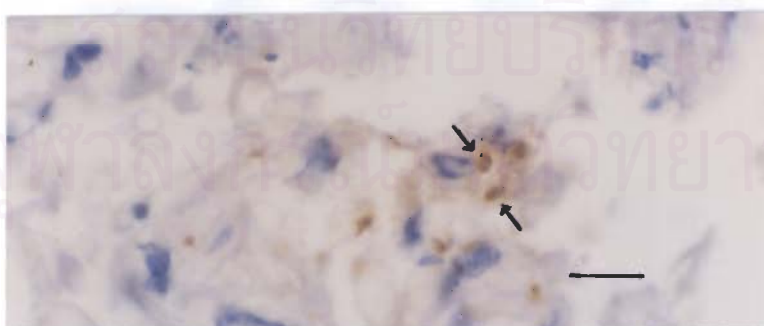
Fig. 6. Scattered *N. caninum* tachyzoites in the placenta. ABC method, hematoxylin counter stain. (bar = 10 μm)
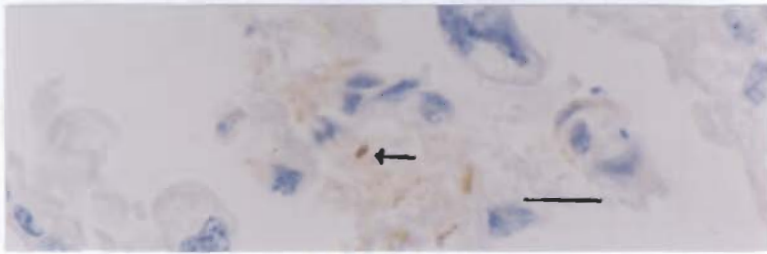
Fig. 7. A single tachyzoite (arrow) of *N. caninum* found in the placenta. ABC method, hematoxylin counter stain. (bar = 12.5 μm)

Fig. 8. A group of tachyzoites of *N. caninum* in goat heart. Positive control tissue. ABC method, hematoxylin counter stain. (bar = 10 μm) (Tissue supplied by McAllister, USA)

Fig. 9. Two *N. caninum* tachyzoites in goat heart. Positive control tissue. ABC method, hematoxylin counter stain. (bar = 12.5 μm) (Tissue supplied by McAllister, USA)
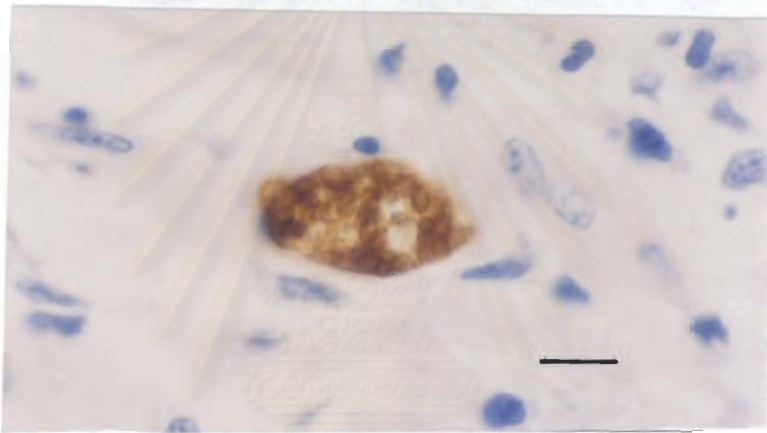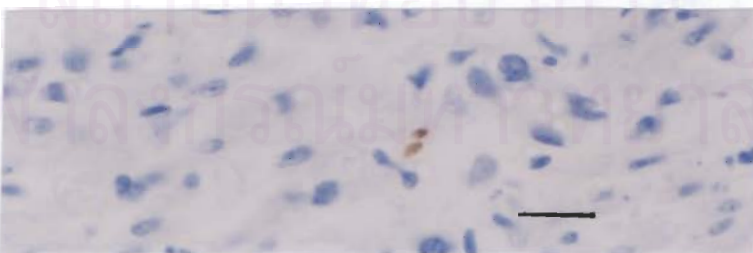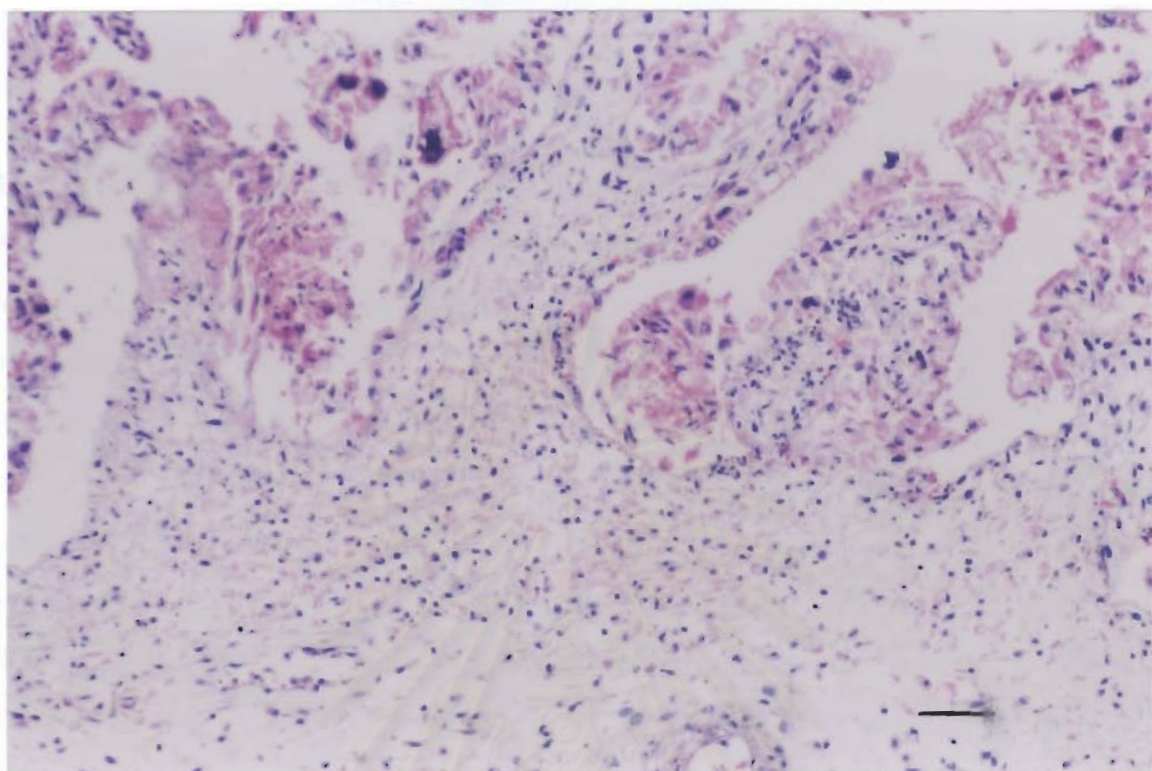
Fig. 10. Nonsuppurative necrosis of the placenta showing mononuclear
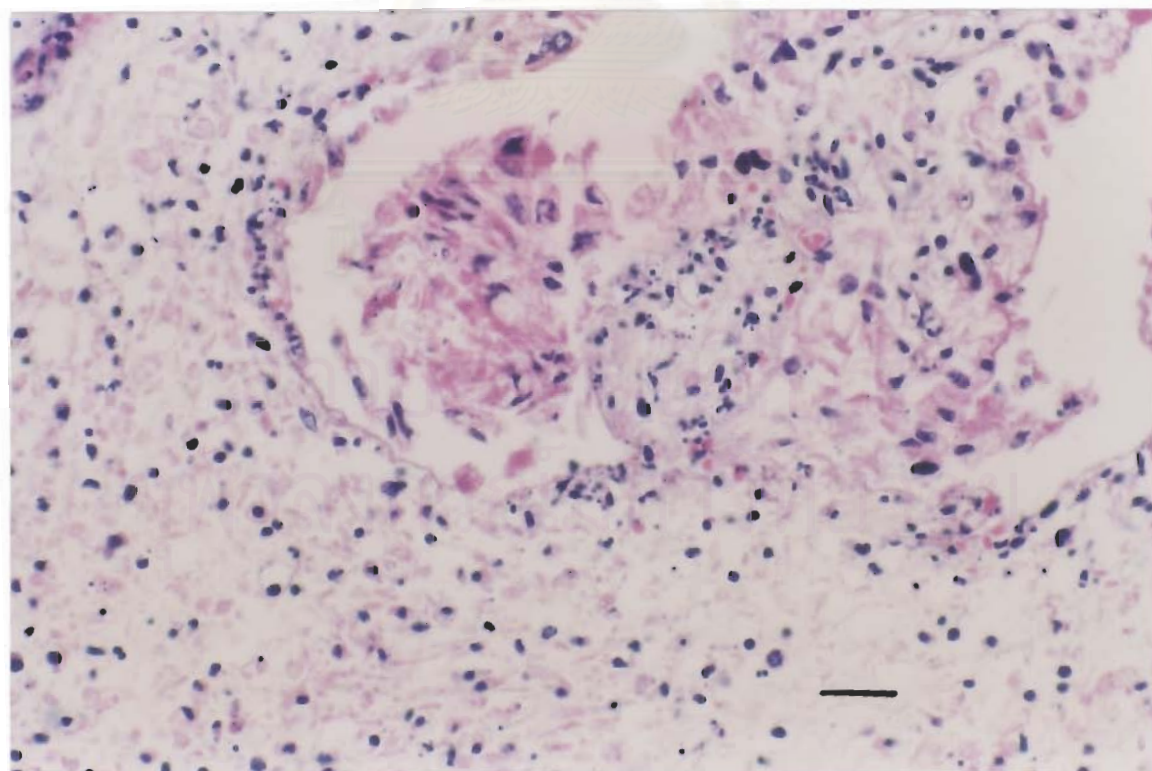
cell infiltration. H & E (bar = 50 μm)

Fig. 11. The same tissue as in Fig. 10 at higher magnification. (25μm)

CHAPTER 5

Discussion

5.1   Seroprevalence study

Although not directly comparable and slightly lower, the seroprevalence of antibodies to *N. caninum* in dairy cattle in Nakhon Pathom province is similar between this study and a previous finding by Suteeraparp *et al*. (1999) (5.46% vs. 6.9%). The extend of infection or exposure among dairy cattle population in this area seems to be stable. However, we found that infection rate of *N. caninum* among dairy herds (33.9%; 20/59) was high in this area. Chanlun (2002) found that 9 out of 11 herds (81.8%) were seropositive, having a within herd seroprevalence range of 0-46%, in Northeast of Thailand. The finding of Chanlun (2002), though the number of herds studied was small, and our result indicate the existence of high infection of *N. caninum* protozoan among dairy herds in these areas.

The seroprevalence may vary widely depending on the location and geographic region. Seroprevalence of cows may be as low as 2% in Sweden (Björkman *et al*., 2000) and as high as 87% in the Netherlands (Wouda *et al.,* 1999a). In France, one report stated that 64% (27/42) of herds and 5.6% (107/1924) of cows were seropositive to *Neospora* (Ould-Amrouche *et al*., 1999). Similarly a herd prevalence of 63.6% was reported in Brazil (de Souza *et al*., 2002). Even higher herd infection (94%) was found in Spain (Mainar-Jaime *et al*., 1999).

Neospora seropositivity in cattle is associated with many risk factors such as the presence on the farm of dogs, cats, poultry, ducks, pigeon, rabbits (Bartels *et al*., 1999; McGuire *et al*., 1999; Ould-Amrouche *et al*., 1999). Particularly, after confirmation of the dog as definitive host (McAllister *et al*., 1998a; Lindsay *et al*., 1999), the presence of dogs on the farm has been assumed to have the most chances of horizontal transmission through ingestion of oocysts shed by infected dogs. Also there are reports stating high prevalence of antibodies to *N. caninum* in farm dogs than that of urban dogs and strong relationship between *N. caninum* infection of farm dogs and cattle

(Sawada et al., 1998; Mainar-Jaime *et al.*, 1999; Wouda *et al.*, 1999b; de Souza *et al.*, 2002). In contrast to these reports the present study showed no evidence of association between herd seropositivity and the presence of dogs on the farm (p>0.05; table 5). In this study 74.5% of farms had dogs, the number ranging from 1 to 7 and 37.2% (16/43) of these farms were seropositive. Surprisingly, only one seropositive dog among 82 farm dogs tested was found and this dog was from a seropositive farm. In one study in France, more than 20% of herds were seropositive to *Neospora* but no seropositive dog was present in 58% (7/12) of seropositive herds (Pitel *et al.,* 2001). It is difficult to explain about the disagreement between the present study and other reports. There are some possible reasons. The first one is the differences in the cut-off values of antibody titers and use of different serological tests. The second is that the dogs in this study might not be infected by *N. caninum* or they did not seroconvert, even though they were infected, or seroconverted but not to a detectable level. McAllister *et al.*, (1998a) and Lindsay *et al.* (1999) reported that one of experimentally *Neospora*-infected dogs in their respective experiments did not seroconvert, even though oocysts were shed, after feeding mouse brain containing *Neospora* tissue cysts. Dijkstra *et al.* (2001) found that none of the dogs fed *Neospora*-infected bovine placentas did not seroconvert but shed oocysts and no renewed oocyst shedding was observed even after repeated ingestions of infected placentas. There are no wild canids, which have also been assumed to be definitive host, in this area. The absence of wild canids and low seroprevalence of dogs suggest the possible existence of other definitive host rather than canids and the detection of *N. caninum* antibodies in the sera of farm dogs in the sero-epidemiological study may need careful interpretation. One thing to be noted is that the accessibility of farm dogs (or stray dogs) among nearby-farms is a considerable factor for the spread of infection in areas where many farms are established very close each other.

The present study showed that 6.1% (24/391) of cows ($\geq$3 year) and 4% (6/150) of heifers (1-2 years) were seropositive to *N. caninum*. The number of seropositive cows was higher than heifers, but there was no statistically significant association between the seropositivity of cows and heifers (p>0.05; table 5). Similarly, Davison *et al.* (1999a), Paré *et al.* (1996) and Pitel *et al.* (2001) reported no significant seroprevalence

differences among the age groups of females in the range of 7 month to 5 years. Although seroprevalence is not different, the level of antibody titers fluctuates in accordance with the situations, particularly reproductive stage of the infected cows (Jenkins *et al*., 1997; Stenlund *et al*., 1999). In a study of 2 consecutive pregnancies in the naturally infected cows, the antibody level was found to be at its peak at 4-5 months before parturition and decreased again 2 months before parturition (Stenlund *et al*., 1999) but Jenkins *et al*. (1997) found high antibody levels early in the gestation. Also, antibody titers of precolostral and colostral calves (up to 6 months) were significantly higher than other age group (Pereira-Bueno *et al*., 2000). Although we could not relate to the reproductive stages, the different antibody levels of the seropositive cows, in this study, were also found with many cows (60% of seropositive cows) having high antibodies (Fig. 2); possibly these cows had had a higher exposure to *N. caninum*.

There was a significant association between the herd size and the seropositivity of the herd (p<0.05). Not many reports have been found with regard to the association between these two parameters. Davison *et al*. (1999a) found that the herd size did not significantly affect the seroprevalence. The disagreement between the present study and their findings is not explainable. It is possible that the vertical transmission may act as a contributing factor in the higher incidence in large farms.

The facts that antibodies to *N. caninum* was detected in 1 of 12 cows (8.3%) with history of abortions and absence of *B. abortus* antibodies in these cows were suspicious of *N. caninum* as a cause of abortion. Suteeraparp et al. (1999) reported that none of the cows in Nakhon Pathom and other provinces showed antibodies against *B. abortus* in their sera. The present study also found that low prevalence of *B. abortus* and no *B. abortus* antibodies in cows with abortion history. These facts show brucellosis as a less significant cause of abortion and overall seroprevalence of *N. caninum*, about 2 times as large as that of *B. abortus* in both individual and herd levels (table 2), indicates the possible potential threat of *Neospora* abortion in Nakhon Pathom Province. On the other hand, it is worth to note that many of congenitally infected fetuses did not terminate in abortion, instead even gave birth to clinically healthy calves (Paré *et al*., 1996; Moen *et*

al., 1998). More clearer picture would have been acheived if the serological tests of other abortificients such as bovine viral diarrhoea virus were included.

5.2   Immunohistochemical study of aborted fetuses

Although seroprevalence reports on *N. caninum* infection are available in Thailand, the parasite has not been identified yet. This report was the first finding of *N. caninum* parasites in the placenta (Fig. 4, 5, 6, and 7) of a seropositive cow aborted at 4 months of gestation. It has been known that the tissue cysts are most commonly found in central nervous system and tachyzoites of the parasite in the heart and liver (Dubey and Lindsay, 1996; Wouda *et al.,* 1998) but in this study we could not find tissue cysts or the organisms in any of these organs. The possible reasons may be that we had a fewer samples or the presence of parasites were very scarce to find. We collected only 2 fetal brains from 5 seropositive cows and one of them was advanced autolysis. It is also reported that only a few *N. caninum* are present in tissues and these are often not visible in H&E stained sections (Lindsay and Dubey, 1989; Dubey and Lindsay, 1996). The autolysis and the rarity of organisms present in the tissues make difficult to easily find in the histologic sections; even the sensitivity of the most efficient method (IHC) to detect *N. caninum* is low (Dubey, 1999a). Dijkstra *et al.* (2001) fed the dogs placentas of *Neospora* infected cows and they found that the dogs shed oocysts but they could not identify the *N. caninum* in the placentas by IHC. Therefore, it is not surprising to see only a few reports on the finding of *N. caninum* organisms in the placenta of infected cows (Shivaprasad *et al.,* 1989; Fioretti *et al.,* 2000; Bergeron *et al.,* 2001).

In conclusion, neosporosis is more common in herd rather than individual level. The indentification of *Neospora* parasite and high incidence of *Neospora*-seropositive aborting cows strongly indicate the possibility of *N. caninum* as a major cause of abortion. Further studies are necessary to investigate the extend and severity of these abortions, epidemiological status and exclusion of other abortificient diseases so that the preventive and control measures can be monitored.

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Appendices

Appendix A

Phosphate buffered saline (PBS), pH 7.4

Stock solution (0.1 M, 10x)

NaCl	40.00 g
KCl	1.00 g
Na_2HPO_4	5.75 g
$K_2H_2PO_4$	1.00 g
DW make to	500 ml

(PBS stock solution is stored at room temperature and

diluted to working buffer (0.01 M) when used).

Appendix B

Citrate buffer, pH 6.0

Stock solution A:	Citric acid	21.10 g
	DW	1000 ml
Stock solution B:	Sodium citrate	29.40 g
	DW	1000 ml
Working citrate buffer solution for		2000 ml
	Solution A	36 ml
	Solution B	164 ml
	DW	1800

(pH adjusted to 6.0 with 1 N NaOH)

Appendix C

Flow chart of IHC staining

1. Cut 4-5 μm sections from formalin fixed, paraffin embedded samples

2. Deparaffinization

   Xylene (3 changes, 5 min each), Xylene+absoloute alchohol (2 min),

   Graduated alcohols (100%, 95%, 80%, 70%; 2 min each),

   running water (5 min), DW (5 min), PBS (5 min)

3. Blocking endogenous peroxidase by 3% H_2O_2, 30 min, at room temperature

4. Wash with DW (5 min), PBS (2 x 5 min)

5. Antigen retrieval

   Put slides into 0.01 M citrate buffer container.

   Heat in microwave (1200W, high power, 5 min)

6. Cool 30 min, wash with PBS (3 x 5 min)

7. Blocking non-specific binding

   Apply 10% bovine serum albumin (BSA),

   incubate at 37°C, in humid chamber, 30 min

8. Wash with PBS (3 x 5 min)

9. Apply primary *N. Caninum* antibody (1:10,000 dilution in PBS),

   kept overnight at 4°C.

10. Wash with PBS (3 x 5 min)

11. Apply biotinylated secondry antibody (goat anti-rabbit IgG, 1:400 dilution in PBS),

    Incubate at 37°C, in humid chamber, 30 min

12. Wash with PBS (3 x 5 min)

13. Apply ABC (A 4 μl +B 45 μl in PBS 5 ml; prepare 30 min before use)

    Incubate at 37°C, in humid chamber, 30 min

14. Wash with PBS (3 x 5 min)

15. Put slides in DAB substrate (0.075g + Tris buffer 150 ml + 30% H_2O_2 50 μl), 8 min

16. Wash in running water, 5 min

17. Counterstain with hematoxylin, 30 sec

18. Wash in running water, 5 min

19. Dehydration

    95% ethanol, 100% ethanol (2 steps), xylene + absolute alcohol, xylene (2 steps):

    2 min each

20. Mount slides with mounting media (DPX)

VITAE

Mr. Than Kyaw, a Myanmar student, was born in central Myanmar in 1951 and obtained his Bachelor of Veterinary Science degree from the Veterinary Institute, Yangon, in 1973. He worked as a demonstrator in the Department of Animal Science at the Institute of Agriculture from 1974 to 1986. He studied at Lincoln University, New Zealand, for his Diploma in Agricultural Science in 1982 and at Melbourne University for his Master of Agricultural Studies emphasizing environmental effect on the performance of layers during 1987-1989. He then worked as assistant lecturer, lecturer and associate professor in the department of Animal Husbandry at the University of Veterinary Science till 2000. In 2001, he was selected as a doctoral student by the Union of Myanmar Government to study ruminant reproduction in the Department of Obstetrics, Gynaecology and Reproduction at the Chulalongkorn University with a financial support by Charoen Pokphand Foods Public Company Limited, Thailand. His research area has been and will cover epidemiology of *Neosporosis* in dairy cattle in Thailand.