THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Veterinary Parasitology)

DEGREE

Veterinary Parasitology

FIELD

Parasitology

DEPARTMENT

TITLE: Epidemiology of *Neospora caninum* Infection in Water Buffaloes in Northeast Thailand

NAME: Miss Chanya Kengradomkij

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Sathaporn Jittapalapong, Ph.D.)

THESIS CO-ADVISOR

(Assistant Professor Burin Nimsuphan, Ph.D.)

DEPARTMENT HEAD

(Associate Professor Sathaporn Jittapalapong, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

EPIDEMIOLOGY OF *NEOSPORA CANINUM* INFECTION IN WATER BUFFALOES IN NORTHEAST THAILAND

CHANYA KENGRADOMKIJ

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Veterinary Parasitology)
Graduate School, Kasetsart University
2014

Chanya Kengradomkij 2014: Epidemiology of *Neospora caninum* Infection in Water Buffaloes in Northeast Thailand. Master of Science (Veterinary Parasitology), Major Field: Veterinary Parasitology, Department of Parasitology. Thesis Advisor: Associate Professor Sathaporn Jittapalapong, Ph.D. 99 pages.

Neospora caninum is an important obligate intracellular protozoa that causes neosporosis in cattle worldwide. Neosporosis is one of the major diseases that have an impact on cattle production such as a reduction of milk yield, an increasing of herd abortion and a culling of animals; therefore, leading to the substantial economic losses. Bovine neosporosis in Thailand has been concerned due to its effect on animal production; however, information of neosporosis in buffaloes in Thailand is very scattered. The objectives of this study were (1) to detect antibodies against *Neospora caninum* infection of water buffaloes in northeast Thailand by using the indirect fluorescent antibody test (IFAT) and (2) to identify factors related with *N. caninum* infections of water buffaloes. In 2010, the sera of 628 water buffaloes from 288 farms in northeastern provinces of Thailand including Ubon Ratchathani, Surin, Buri Ram, Si Sa Ket, Sakon Nakhon, and Roi Et were collected and tested by IFAT. An overall positives to *N. caninum* were 9.1% (57/628) and was ranged between 3.9% and 16.7% among six provinces and Ubon Ratchathani had the highest individual prevalence at 16.7% (23/138). The southern part of northeast Thailand had the higher infection 13.2% compared to the northern part (4.2%). The total herd prevalence was 16.7% (48/288) and Si Sa Ket had the highest farm prevalence (36.4%, 8/23). The herd size was found that the farm with more than 5 animals/farm size had higher prevalence (30.0%) than another group (16.2%). Water buffaloes with > 10 years old (16.1%) had the statistically higher prevalence than buffalo with 5-10 years (13.4%), 3-5 years (9.2%), and less than 3 years (1.2%) ($p<0.001$). However, there were no significant differences found regarding sexes.

/ /

Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

For the accomplishment of my research, I am sincerely indebted to my committee members,

To Assoc. Prof. Dr. Sathaporn Jittapalapong, my advisor, for leading me into this field. Be grateful for his kind, patience, support, suggestion for better writing of thesis and allowing me to practice and develop the laboratory skill. His benevolence leads me to an achievement of the research.

To Assist. Prof. Dr. Burin Nimsuphan , my co-advisor, for indispensable suggestion, kindness and great support. My research is not complete if lack of the valuable advice from him.

To Assist. Prof. Dr. Sirichai Wongnakpech, for kind-heartedness, encouragement and helpful for statistical analysis. The statistical advice from him has fulfilled the achievement of my research.

The study was plentiful facilities provide at the Section of Protein Laboratory and Cell Culture Laboratory, Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University. I would like to thank the staff at Department of Parasitology, Faculty of Veterinary medicine, Kasetsart University and laboratory colleagues whom gave me for the generous help in everything and kindness suggestions. I do thank to my graduated friends from Veterinary Parasitology, for their friendship, help, cheerfulness and great support.

Finally, I truthfully thank my family who always gave me the chance to do everything, wish well, perpetual support and encouragement.

Chanya Kengradomkij

September 2014

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure **Page**

Appendix Figure

LIST OF ABBREVIATIONS

AU$	=	Australian dollar
CO_2	=	Carbon dioxide
CNS	=	Central nervous system
X^2	=	chi-square
cELISA	=	Competitive Enzyme-Linked Immunosorbent Assay
CI	=	Confidence Interval
°C	=	Degree(s) Celsius
df	=	Degrees of freedom
DNA	=	Deoxyribonucleic acid
ELISA	=	Enzyme Linked Immunosorbent Assay
EMs	=	Electron Microscopes
et al.	=	et. alii (and others)
e.g.	=	for example
FBS	=	Fetal bovine serum
G	=	Gravity
HE	=	Hematoxylin and eosin stain
i.e.	=	that is
IHC	=	Immunohistochemistry
IFAT	=	Indirect fluorescent antibody test
kg.	=	Kilogram
µl	=	Microliter(s)

LIST OF ABBREVIATIONS (Continued)

μm	=	Micrometers
mm	=	Millimeters
MAT	=	Modified agglutination test
NZ$	=	New Zealand dollar
NAT	=	Neospora agglutination test
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
P	=	P value
spp.	=	species
T. gondii	=	*Toxoplasma gondii*
UK	=	United Kingdom
US$	=	United States dollar
USA	=	United States of America
$	=	Dollar

EPIDEMIOLOGY OF *NEOSPORA CANINUM* INFECTION IN WATER BUFFALOES IN NORTHEAST THAILAND

INTRODUCTION

Neospora caninum is an important obligate intracellular protozoa that infects a wide variety of mammals and causes neosporosis. *N. caninum* was first reported as an unidentified protozoa in 1984 (Bjerkas *et al*., 1984), later described, and named by Dubey *et al*. (1988a). In retrospective studies, *N. caninum* is now classified in the family Sarcocystidae within the phylum Apicomplexa. Evidences of *Neospora*-infections have been reported in many domestic and wild animals and many reports have been emerged suggesting that neosporosis is one of the major causes of abortion in cattle worldwide (Dubey and Lindsay, 1996; Dubey, 1999b; Anderson *et al*., 2000; Hemphill and Gottstein, 2000; Reichel, 2000; Antony and Williamson, 2001). Neosporosis affects cattle production by causing abortion, stillbirths, and the birth of weak calves (Kyaw *et al*., 2004). Bovine abortions due to this parasite could result in a reduction of milk production and culling of animals leading to the substantial economic loss (Hobson *et al*., 2002; Romero *et al*., 2005; Thurmond and Hietala, 1996). The estimated economic loss per year due to neosporosis in dairy cattle in New Zealand (NZ$ 17.8 million), Australia (AU$ 85 million for dairy and 25 million for beef cattle) were reported by Reichel (2000) and in California, USA (US$ 35 million) by Dubey (1999a). Thailand has a cattle population of 6.95 million (0.53 million dairy and 6.42 million beef) and 1.19 million buffaloes (Department of Livestock Development [DLD], 2010). These animals are involved in an important economic sector of the country, through milk and meat production as well as a draft animal for agricultural practice.

N. caninum has been recognised not only as a primary cause of bovine abortion throughtout the world, but also as a causative agent of neuromuscular disease in dogs (McInnes *et al*., 2006). Neosporosis was first described in dogs in Norway in the mid-1980's as causing neuromuscular degeneration leading to hind limb paralysis

and encephalomyelitis (Bjerkas *et al*., 1984; Dubey *et al*., 1988a). Dogs have recently been identified as a definitive host, whereas other vertebrates may act as intermediate hosts (McAllister *et al*., 1998; Lindsay *et al*., 1999). Dogs and cattle are known as the most susceptible species, whereas information on neosporosis in buffaloes is very scattered. Buffaloes are an important natural host of *N. caninum* (Rodrigues *et al*., 2004). A few reports have been shown the seroprevalence of *N. caninum* infection in water buffaloes in many countries, such as Egypt (Dubey *et al*., 1998b), Vietnam (Huong *et al*., 1998), Italy (Guarino *et al*., 2000), Brazil (Gennari *et al*., 2005) and China (Yu *et al*., 2007).

Buffaloes have been an integral part of agricultural process in Asia producing draft power, milk, meat, and hides. Currently, 194 million buffaloes provide 92 million tonnes of milk, 3 million tonnes of meat, and 0.9 million tonnes of hide in the world and 97% of buffaloes (approximately 188 million animals) were in Asia (Food and Agriculture Organization [FAO], 2010). Unfortunately, however, buffaloes did not receive the attention of the policy makers and the researcher in accordance with their merits, which resulted in buffalo population decline in several eastern Asian countries.

In Thailand, buffaloes have been significantly exploited for various purposes, recognized to contribute the sustainability of mixed crop-livestock farming systems, and increased farmer's income and food security in Asia. However, their usages have been neglected by least research, development, and promotion. These will lead to less health care of buffaloes. Most buffaloes in Thailand are not vaccinated against certain infectious diseases. In general, most buffaloes are belong to small farm holder who prefer to use traditional curing methods than using modern medicine.

In Thailand, the information of neosporosis in water buffalo is limited since there were a few reports (Wiengcharoen *et al*., 2010; Nam *et al*., 2012). Therefore, there is very urgent need to have more investigation to use to establish the control program of neosporosis in buffaloes.

OBJECTIVES

1. To detect antibodies against *Neospora caninum* infection of water buffaloes in northeast Thailand by using IFAT.

2. To identify factors related with *N. caninum* infections of water buffaloes.

LITERATURE REVIEW

1. The overview of *Neospora caninum*

1.1 Morphology of *N. caninum*

N. caninum has a heteroxenous life cycle. Dogs (*Canis familiaris*) and coyotes (*Canis latrans*) are the only recognized definitive hosts (McAllister *et al*., 1998; Gondim *et al*., 2004b). Cattle and a wide range of other warm-blooded animals can act as intermediate hosts. *N. caninum* has three infectious stages including tachyzoites, bradyzoites, and sporozoites.

Tachyzoites and bradyzoites (Figure 1) invade tissues of infected hosts (intermediate and definitive) while sporozoites are inside oocysts that are excreted in the faeces of the definitive host. Tachyzoites (Figure 1A-C) are lunate-shaped, approximately 2x6 µm with a central nucleus but it lack of amylopectin granules (unlike bradyzoites). Tachyzoites rapidly divide within cells and infect many cell types including neural cells, vascular endothelial cells, myocytes, hepatocytes, renal cells, alveolar macrophages, and placental trophoblasts (Barr *et al*., 1991b; Dubey *et al*., 2002).

Tachyzoites are located within the host cell's cytoplasm with or without a parasitophorous vacuole (PV). Varied from many to none of intravacuolar tubules may be found in the PV. Tachyzoites of *N. caninum* have typical organelles as same as *T. gondii*'s tachyzoites. Tachyzoites have a three-layered plasmalemma, 22 subpellicular microtubules, 2 apical rings, 1 conoid, 1 polar ring, mitochondria, up to 150 micronemes, between 8 and 18 rhoptries (some extending posterior to the nucleus), a Golgi complex, rough and smooth endoplasmic reticulum, 1 nucleus and 1 nucleolus (Speer and Dubey, 1989; Lindsay *et al*., 1993). The rhoptries contain solid electron-dense material and are 2-4 times thicker than the diameter of the micronemes. Micropores have not been seen in tachyzoites in animals but have been

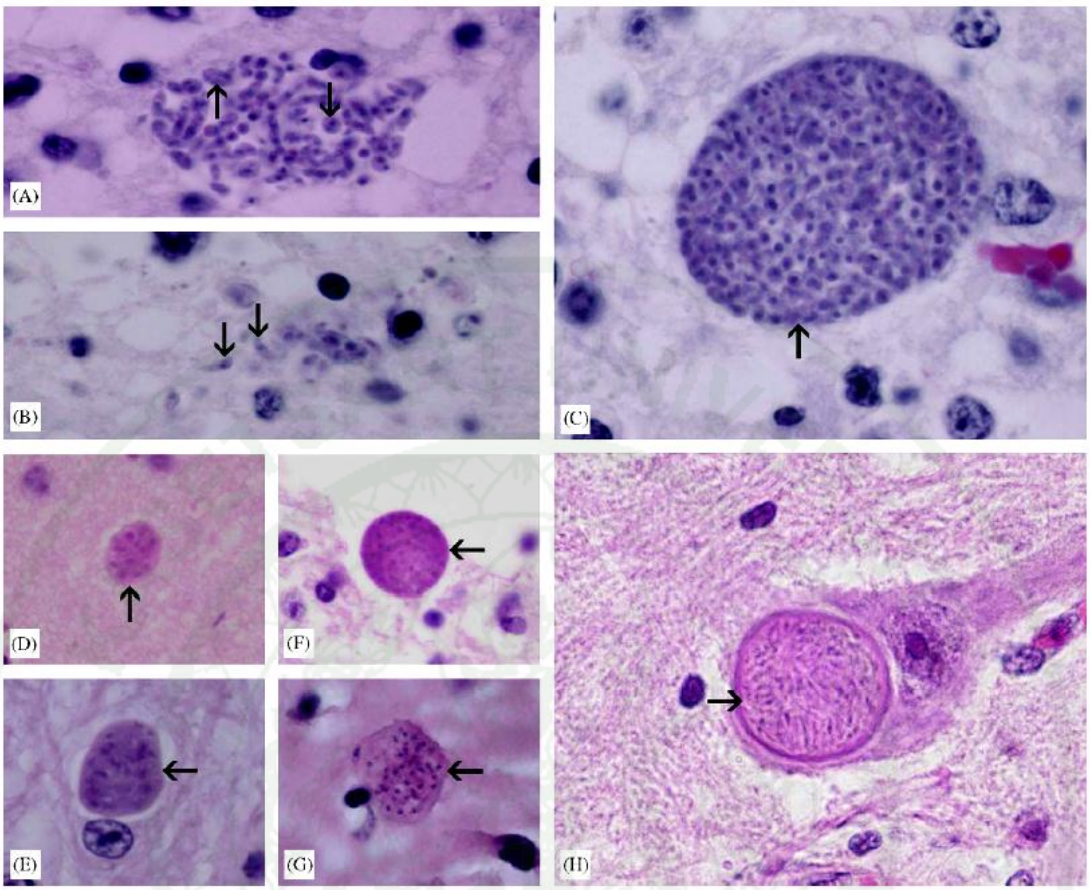


Figure 1 Morphology of *N. caninum*. A-H. *N. caninum* tachyzoites (A-C) and tissue cysts (D-H) as seen in sections of brain and spinal cord of cattle. (A) A group of tachyzoites apparently free in the brain of a fetus. Note dividing tachyzoites (arrows). (B) Extracellular crescentic forms (arrows), rarely seen in sections. (C) A large group of apparently intracellular tachyzoites (arrow). (D-G) Small tissue cysts (arrows) with varying thickness of the cyst wall in brains of aborted fetuses. (H) A thick walled (arrow) tissue cyst within a neuron in the spinal cord of a 3-day old calf. Hematoxylin and eosin stain (HE). x600.

Source: Dubey *et al*. (2006)

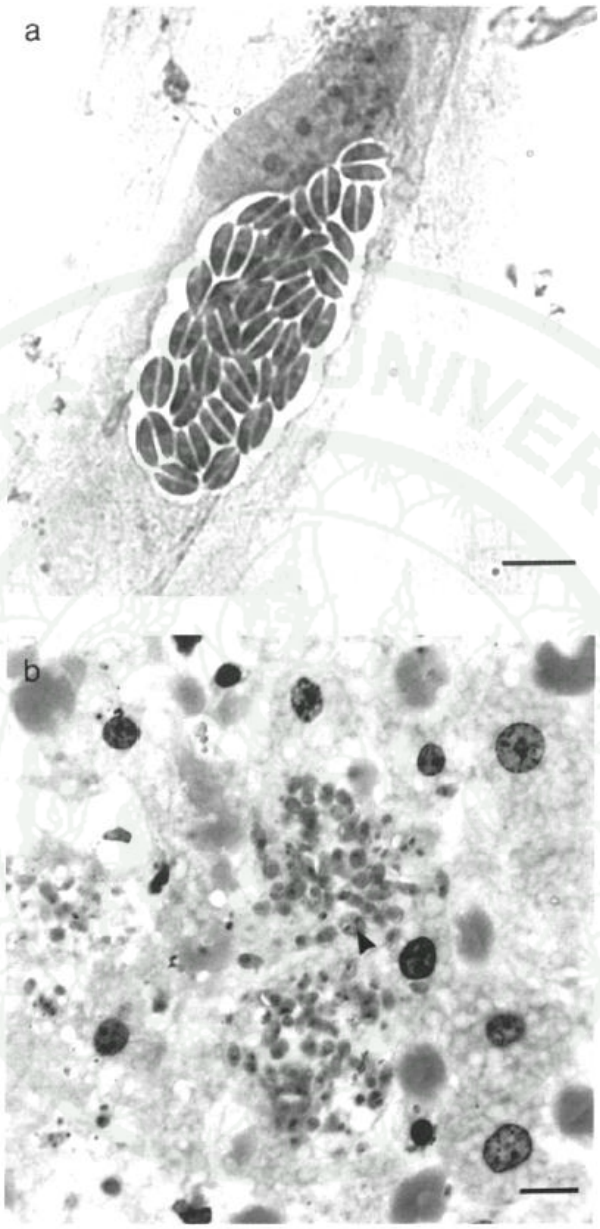


Figure 2 Tachyzoites of *N. caninum*. A group of tachyzoites in a human foreskin fibroblast cell (a) grown in culture and stained with Giemsa is shown. Note that organisms are found in pairs as a result of endodyogeny. Intracellular and extracellular tachyzoites in a section of liver from an experimentally infected rat are shown in (b). Arrowhead point to a dividing organism (HE). Scale bars = 10 µm.

Source: Dubey and Lindsay (1993)

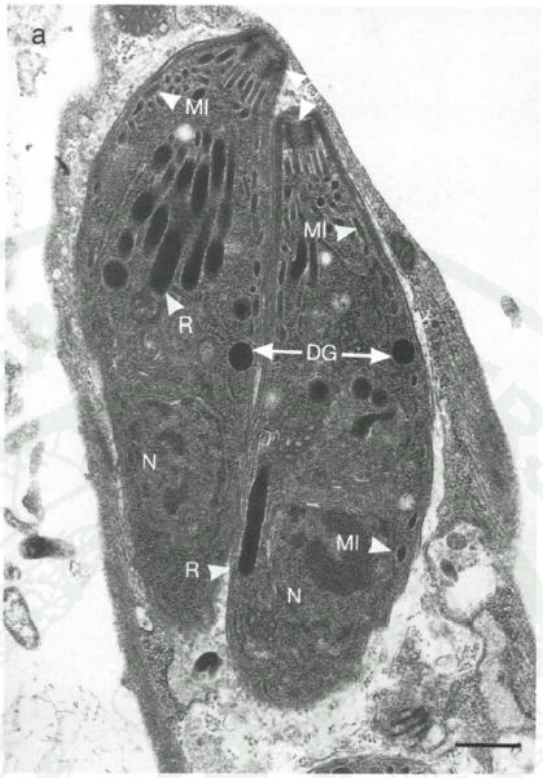


Figure 3 Tachyzoites of *N. caninum* in human foreskin fibroblast cell cultures. Two *N. caninum* (NC-I strain) tachyzoites, one day post-inoculation (a). Note several rhoptries (R) with electron-dense contents anterior to the nucleus and one rhoptry posterior to the nucleus in *N. caninum* and two anterior rhoptries. Also, note the micronemes (MI), dense granules (DG), nucleus (N) and mitochondria (MT) in the tachyzoites. Scale bars = 0.5 µm.

Source: Dubey and Lindsay (1993)

found in tachyzoites grown in cell culture (Speer and Dubey, 1989; Lindsay *et al*., 1993).

Bradyzoites are slowly replicated as encysted stages of the parasite. Tissue cysts may vary considerably in size depend on the number of bradyzoites (Figure 1 D-H). In dogs tissue, cysts up to 107 µm in diameter with a cyst wall up to 4 µm thick

have been recorded (Dubey *et al*., 2002). In bovine fetuses and congenitally infected calves (Figure 1), tissue cysts are found in the brain and spinal cord and rarely more than 50 µm in diameter with a cyst wall usually less than 2.5 µm thick (Dubey *et al*., 1989; Barr *et al*., 1991b). A few thin-walled tissue cysts have been reported in skeletal muscles of two naturally infected 2-day calves (Peters *et al*., 2001a). A definitive carnivore host can be acquired the infection by ingestion of tissues containing cysts. Bradyzoites are slender and approximately 6.5x1.5 µm (Dubey *et al*., 2004), with a terminally located nucleus (Figure 4), and contain a few amylopectin granules stained red with the periodic acid Schiff reaction. A single tissue cyst (11x9 µm) was found in the brain of a 32-day fetus after inoculation of the dam with *N. caninum* (Dubey *et al*., 1992b). A tissue cyst-like structure was also found in a histopathological section of brain at a 14-days fetus after infection of the dam (Macaldowie *et al*., 2004). In tissue cysts, however, it is difficult to identify the stage of the parasite such as bradyzoites and tachyzoites in haematoxylin and eosin (HE) stained sections, because in some cases, *N. caninum* can form a large group of tachyzoites in the tissue cyst (Dubey *et al*., 2002). Bradyzoites can be definitively distinguished from tachyzoites by immunohistochemical labelling with a bradyzoite-specific antibody (McAllister *et al*., 1996). The bradyzoite stage (tissue cysts) is believed that it is found in the tissues of adult cattle, although tissue cysts have not yet been found in naturally infected adult cattle. However, *N. caninum* has been isolated from the brains of two clinically normal cows that had produced infected progeny (Sawada *et al*., 2000; Okeoma *et al*., 2004b).

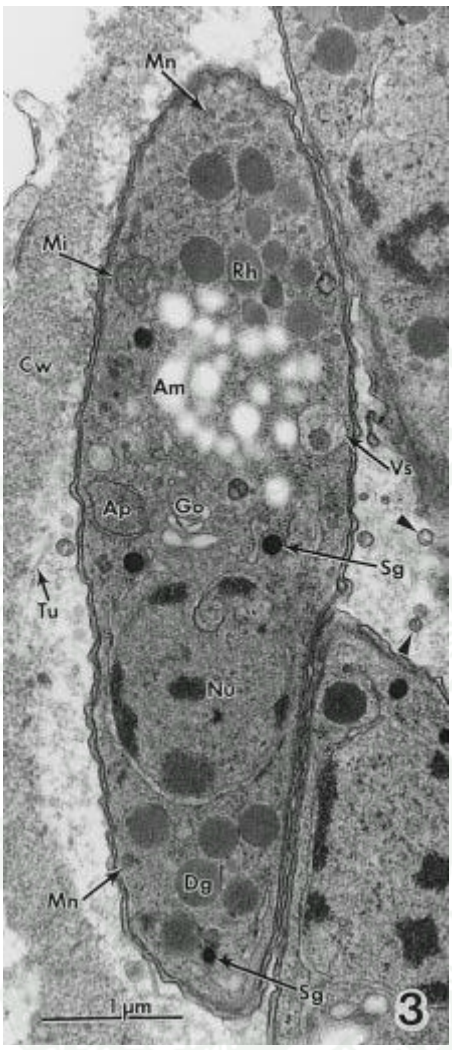


Figure 4 Transmission EMs of bradyzoites of *N. caninum* NC-Liverpool. The nucleus (Nu) is located subterminally and the cytoplasm behind the nucleus contains micronemes (Mn), six dense granules (Dg) and a small dense granule (Sg). Tubules (Tu) project from the inner surface of the cyst wall (Cw) and vesicles (Vs) are scattered among the bradyzoites. Abbreviations: Am, amylopectin; Ap, plastid; Go, Golgi complex; Mi, mitochondrion.

Source: Speer *et al*. (1999)

N. caninum's oocysts, approximately 10x12 µm, are excreted in the unsporulated form in canine feces, and sporulation will later be occurred. Each oocyst contains two sporocysts, and each of which contains four sporozoites, individually

6.5x2 µm (Lindsay *et al*., 1999). Experimentally, dogs have sheded oocysts after ingesting naturally infected tissues from cattle (Dijkstra *et al*.,2001), water buffalo (Rodrigues *et al*., 2004), and white-tailed deer (Gondim *et al*., 2005), but *N. caninum* oocysts have been identified in only the feces of a few naturally infected dogs (Basso *et al*., 2001b; Slapeta *et al*., 2002; McGarry *et al*., 2003). From the past, little was known of the frequency of shedding by canids of *N. caninum* oocysts in nature and of their viability, although dogs were shown to shed oocysts on more than one occasion by McGarry *et al*. (2003). However, seroepidemiological data indicated the importance of the dog in the life cycle of *N. caninum* (Pare *et al*., 1998; Sawada *et al*., 1998; Bartels *et al*., 1999; Mainar-Jaime *et al*., 1999; Ould-Amrouche *et al*., 1999; Wouda *et al*., 1999; Basso *et al*., 2001b; Dijkstra *et al*., 2002; Schares *et al*., 2004; Hobson *et al*., 2005; Rinaldi *et al*., 2005). The schizogonic and gametogenic stages that are presumed to precede the formation of oocysts in the intestines of dogs have not yet been observed, although schizont-like stages have been reported in cell cultures seeded with bradyzoites isolated from the brains of naturally infected dogs (Dubey *et al*., 2004).

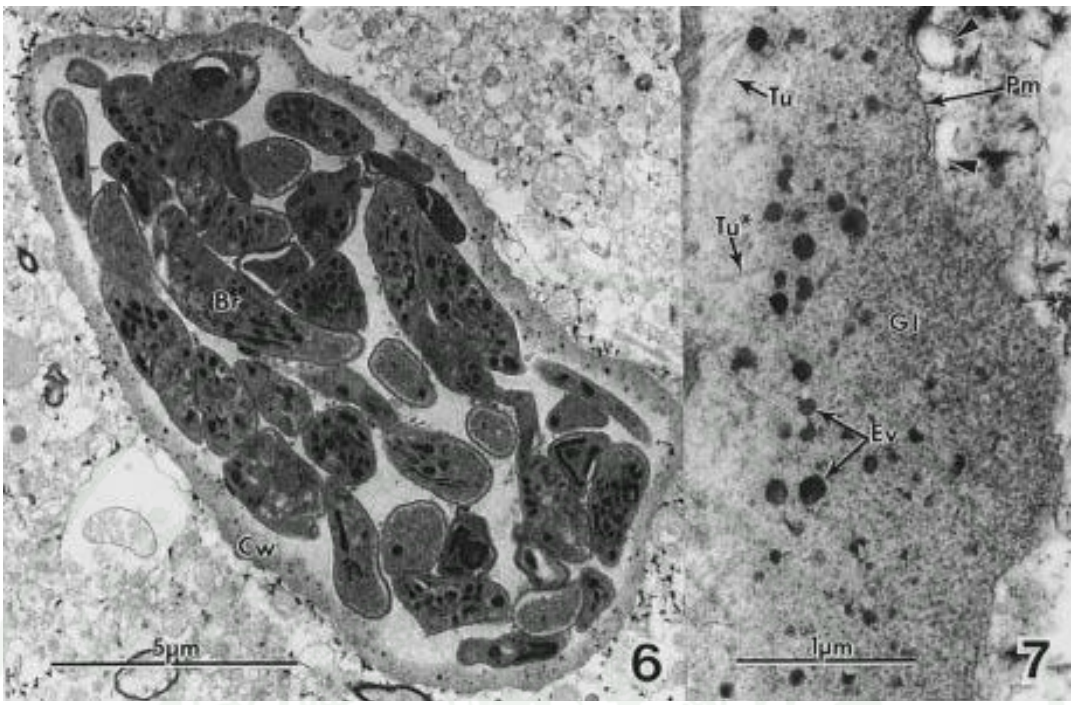


Figure 5 Transmission EM of *N. caninum* tissue cysts. (6) Tissue cyst of *N. caninum* NC-5 showing approximately 30 bradyzoites (Br) surrounded by an irregularly shaped cyst wall (Cw). (7) High magnification of portion of cyst wall in (6), showing electron-dense vesicles (Ev) of various sizes embedded in the granular layer (Gl) and large (Tu) and small (Tu*) tubules projecting from the inner aspect of the cyst wall. Host cell endoplasmic reticulum with distended cisternae (arrowheads) is closely associated with the parasitophorous vacuolar membrane (Pm).

Source: Speer *et al*. (1999)

1.2 Life cycle of *N. caninum*

N. caninum is a coccidian parasite with a wide host range. In general, it is very similar in structure and life cycle to *T. gondii*. Neosporosis is primarily a disease of cattle, and dogs and related canids are definitive hosts of *N. caninum*, while toxoplasmosis is primarily a disease of humans, sheep, and goats, and felids are the only definitive hosts of *T. gondii* (Dubey *et al*., 2007).

The life cycle contains three stages: tachyzoites, tissue cysts, and oocysts (Figure 6 and 7). Tachyzoites and tissue cysts are the stages found in intermediate host cells (Dubey *et al*., 2002). Tissue cysts are found primarily in the central nervous system and other tissues, especially muscles (Peters *et al*., 2001a; Dubey *et al*., 2004). The oocyst, the environmentally resistant stage of the parasite, is excreted in the feces of dogs and coyotes as an unsporulated stage (McAllister *et al*., 1998; Lindsay *et al*., 1999; Gondim *et al*., 2004b). Oocysts will be sporulated outside the host within 24 hours (Lindsay *et al*., 1999).

All stages of *N. caninum* (tachyzoites, bradyzoites, and oocysts) are involved in the transmission of the parasite. The dogs and other canids (foxes) can serve as a definitive host that can produce oocysts (Dubey *et al*., 2007). Canids can be infected by eating tissues of animals that have muscle stages (tissue cysts or bradyzoites) of *N. caninum*. Once in the gut, the parasites are activated and burst out of the tissue cysts to start the life cycle which leads to produce and excrete oocyst into feces. Cattle are infected by ingestion of oocysts via contaminated food, pasture, and water (Dubey *et al.*, 2007). Transplacental infection can be occurred when tachyzoites are transmitted from an infected dam to her fetus during pregnancy (Dubey *et al*., 2007). This route is congenital transmission by transfer of the rapidly multiplying tachyzoites, from the mother across the placenta, to the fetus. Although abortion can occur after tachyzoites infect the fetus, calves are born with no clinical signs of neosporosis. However, these infected calves are capable of transmitting the parasite to their offsprings.

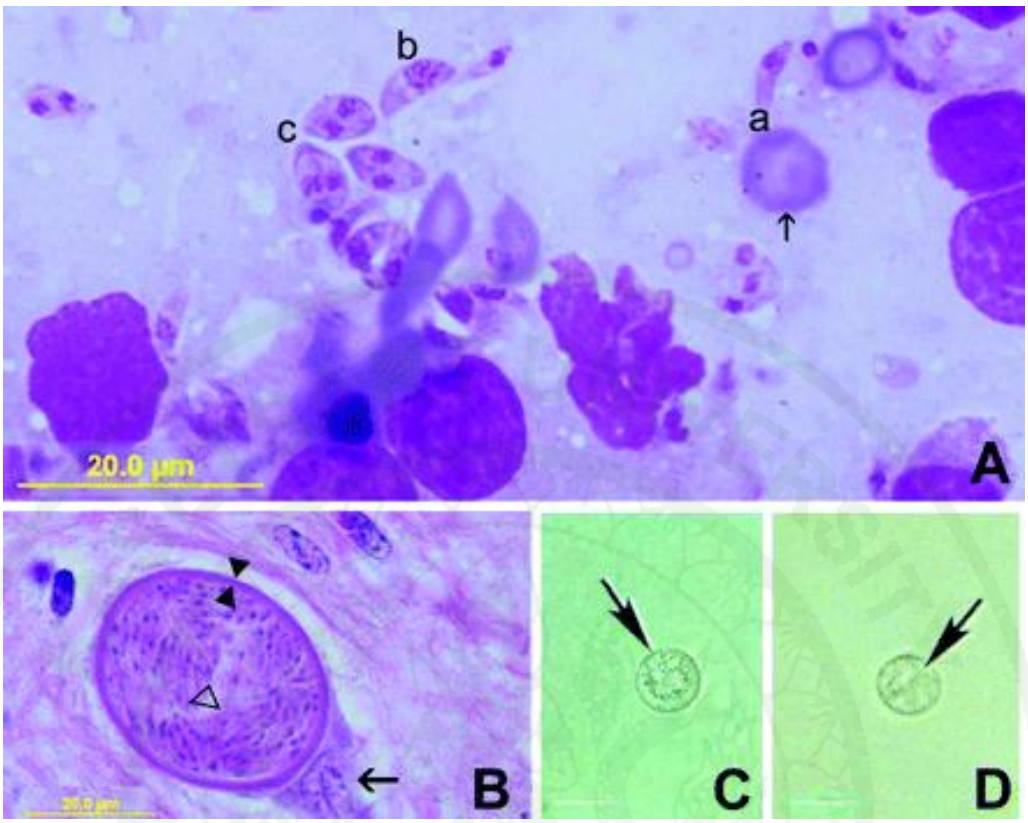


Figure 6 Stages of *N. caninum*. (A) Impression smear of the liver of an experimentally infected mouse depicting numerous tachyzoites (Giemsa stain). The tachyzoites vary in dimension, depending on the stage of division: (a) a slender tachyzoite, (b) a tachyzoite before division, and (c) three dividing tachyzoites compared with the size of a red blood cell (arrow). (B) Histological section of a tissue cyst inside a neuron in the spinal cord of a congenitally infected calf (hematoxylin and eosin stain). The thick cyst wall (opposing arrowheads) enclosing slender bradyzoites (open triangle). The host cell nucleus (arrow) is cut at an angle. (C) Unsporulated oocyst (arrow) with a central undivided mass in the feces of a dog (unstained). Bar = 10 µm. (D) Sporulated oocyst (arrow) with two internal sporocysts (unstained). Bar = 10 µm.

Source: Dubey *et al*. (2006)

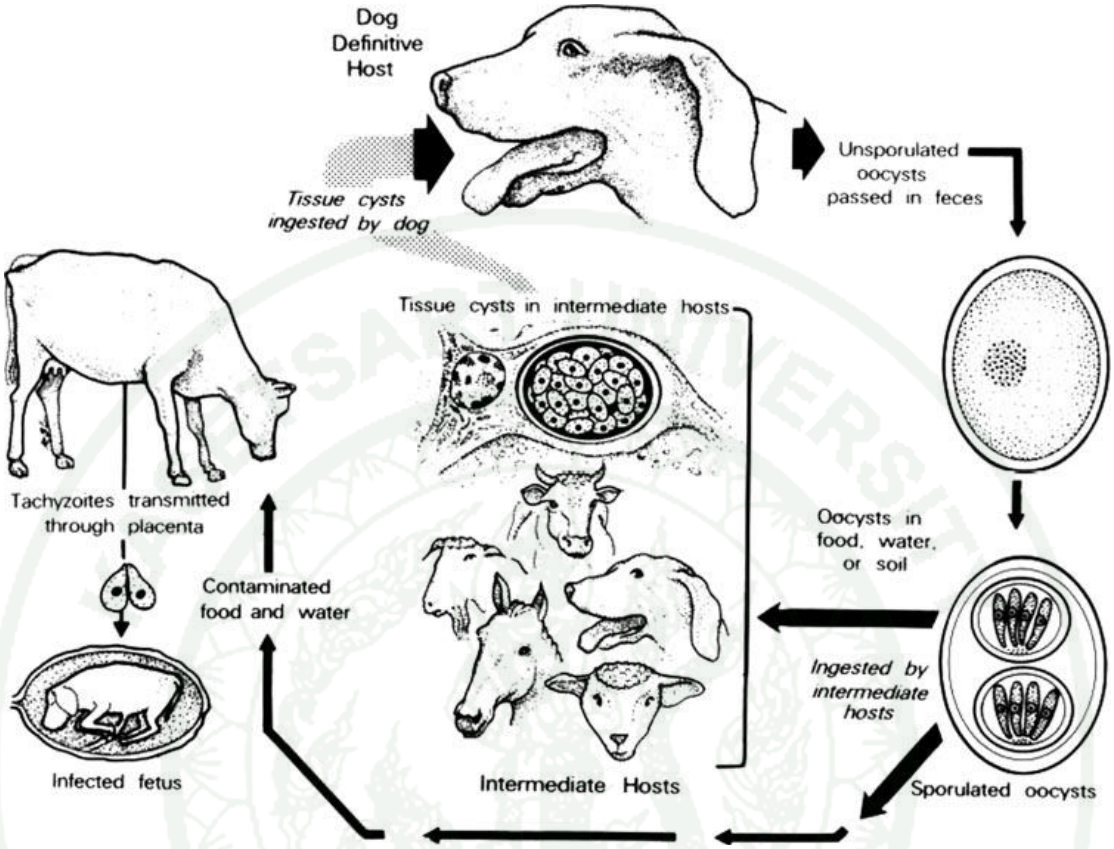


Figure 7 Life cycle of *N. caninum*.

Source: Dubey (2003)

2. Transmission of *N. caninum* in cattle

N. caninum can be postnatally transmitted (horizontal transmission) by ingestion of tissues infected with tachyzoites or tissue cysts or by ingestion of food or drinking water contaminated by sporulated oocysts. Or it can be transplacentally transmitted (vertical or congenital transmission) from an infected dam to her fetus during pregnancy. Exogenous transplacental transmission occurs after a primary, oocyst-derived, infection of the pregnant dam, while endogenous transplacental transmission occurs in the persistently infected dam after reactivation (recrudescence) of the infection during pregnancy. Mice were infected successfully by oral inoculation

of tachyzoites or bradyzoites (Lindsay and Dubey, 1990). These results are of interest because tachyzoites treated with acidic pepsin were rendered noninfective for cell cultures, whereas bradyzoites survived the acidic pepsin (Lindsay and Dubey, 1990). Tissue cysts and bradyzoites can survive up to 2 weeks at refrigeration temperature (4°C) but are killed by freezing (Lindsay *et al*., 1992; Dubey *et al*., 2004). Oocysts were orally infective to cattle (Trees *et al*., 2002; Gondim *et al*., 2004a), goats, sheep (Schares *et al*., 2001), and rodents such as mice, gerbils (*Meriones unguiculatus*), and guinea pigs (*Cavia porcellanus*) (McAllister *et al*., 1998; Dubey and Lindsay 2000; Schares *et al*., 2001). Transplacental transmission has been induced experimentally in cattle, dogs, sheep, goats, monkeys, cats, and mice and occurs naturally in many hosts (Dubey and Lindsay, 1996). Transplacental transmission occurs when tachyzoites from the dam cross the placenta. The ingestion of oocysts is the only demonstrated mode for postnatal (horizontal) transmission in herbivores (Dubey *et al*., 2007).

Transplacental (vertical) transmission

N. caninum is one of the most efficiently transplacentally transmitted parasites among all known microbes in cattle. In some herds, virtually all calves are born infected but asymptomatic. Currently, vertical transmission of *N. caninum* is the only potential route of transmission and the major transmission in dairy herds. In previous studies of a limited number of herds, a rate of vertical transmission varied from 72% to 93% (Pare *et al*., 1996; Schares *et al*., 1998; Wouda *et al*., 1998). The vertical transmission is mainly livestock problem because the most infected cattle and buffaloes were found by this method (Barber and Trees, 1998). The tachyzoites from dam transfer to her fetus throught the placenta causing the infected calf with weak or neurogenic sign or asymtomatic or abortion. A strong evidence for transplacental transmission of *N. caninum* has been obtained by comparison of seroprevalence in dams and their progeny. In cattle and other ruminants, there are no transfer of antibodies from the dam to the fetus, and their placenta has been damaged by an infectious process (Dubey *et al*., 1987). Therefore, detection of specific antibodies from precolostral serum indicates that the fetus produced antibodies by itself. However, no finding of antibody in the fetus are inconclusive of the absence of

infection, because the fetus might have been infected late in gestation, leaving insufficient time for antibody synthesis. Rarely, it is possible for a seronegative dam to give birth to a seropositive calf since the cows have been infected for some time and the declined level of antibodies at the undetectable level (Sager *et al*., 2001; Lopez-Gatius *et al*., 2004; Frossling *et al*., 2005).

Post-natal (horizontal) transmission

The ingestion of sporulated *N. caninum* oocysts from the environment is the only demonstrated natural mode of infection in cattle after birth (Trees *et al*., 2002; Gondim *et al*., 2004a). The unsporulated oocysts are excreted in feces by the definitive host. The sporulation occurs in the environment and the intermediate host is infected by taking food or drinking water contaminated with oocyst. This route is not a major problem in livestock because the amount of oocyst in feces was low (Lindsay *et al*., 1999). To date, cow-to-cow transmission of *N. caninum* has not been observed. At present there is no evidence that live *N. caninum* is present in excretions or secretions of asymptomatic cows. Neonatal calves become infected after ingestion of milk contaminated with tachyzoites (Davison *et al*., 2001), and *N. caninum*-DNA in milk, including colostrum, has been demonstrated (Moskwa *et al*., 2003; Moskwa *et al*., 2007). However, there is inconclusive evidence that lactogenic transmission of *N. caninum* occurs in nature (Dijkstra *et al*., 2001).

Venereal transmission may be possible, but unlikely, as evidenced recently in heifers experimentally infected by intrauterine inoculation of semen contaminated with tachyzoites (Serrano *et al*., 2006), and a dose response has been observed in a titration experiment with seroconversion and maintained antibody levels in heifers inoculated with semen contaminated with 5×10^4 tachyzoites (Serrano *et al*., 2007). Although *N. caninum* DNA has been found in the semen of naturally exposed bulls (Ortega-Mora *et al*., 2003; Caetano-da-Silva *et al*., 2004; Ferre *et al*., 2005), results suggested that viable organisms, if present, were few and infrequent. Additionally, cows inseminated with frozen and thawed semen contaminated with *N. caninum* tachyzoites failed to acquire infection (Canada *et al*., 2006).

3. Clinical signs

3.1 Dogs

N. caninum has been found in naturally infected dogs in several geographical areas, including the USA, Canada, Scandinavia, Europe, Australia, Switzerland, South Africa and Japan (Dubey, 1992). The most cases of clinical neosporosis in dogs were congenitally infected since young animals (Barber *et al*., 1996; Patitucci *et al*., 1997; Reichel, 1998). The most severe and frequent infections have been found in young dogs (<6 months) that frequently show the severe ascending paralysis of the hind limbs more than the front legs (Dubey, 1993; Dubey *et al*., 2009) (Figure 8). Paralysis progresses to rigid contracture of the muscles of the affected limb. The disease may be localized or generalized and virtually all organs may be involved. An unusual sign of neosporosis is dermatitis reported in six dogs (Dubey *et al*., 1988b, 1995; Fritz *et al*., 1997; Perl *et al*., 1998; Poli *et al*., 1998) and these dogs have involved on immunosuppressed condition (Dubey *et al*., 1988b, La Perle *et al*., 2001). Other dysfunctions include difficulty in swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy and heart failure. Non-neurological clinical signs are also related to the cells parasitized, which include the vascular endothelium, myocytes and dermal cells (Dubey *et al*., 1988b). In some pups, joint deformation and genu recurvatum may develop. Cervical weakness, dysphagia, megaesophagus, and ultimately death can occur. Occasional cases may present with signs associated with the heart, lungs or skin (Figure 9). Pancreatitis, hepatitis, or adrenitis may also occur since *N. caninum* tachyzoites have produced necrosis in such organs, resulting in clinical signs such as vomiting and polydipsia as complications of 'neuromuscular cases' (Barber, 1998). In some dogs, the progression may become static, which do not develop severe intracranial manifestations and maintain alert attitudes. They can survive for months with hand feeding and care but remain paralyzed with associated complications. Older dogs, which are less commonly affected, often have signs of multifocal CNS involvement or polymyositis and less common manifestations of myocarditis, dermatitis, pneumonia, or multifocal dissemination. Death can occur in

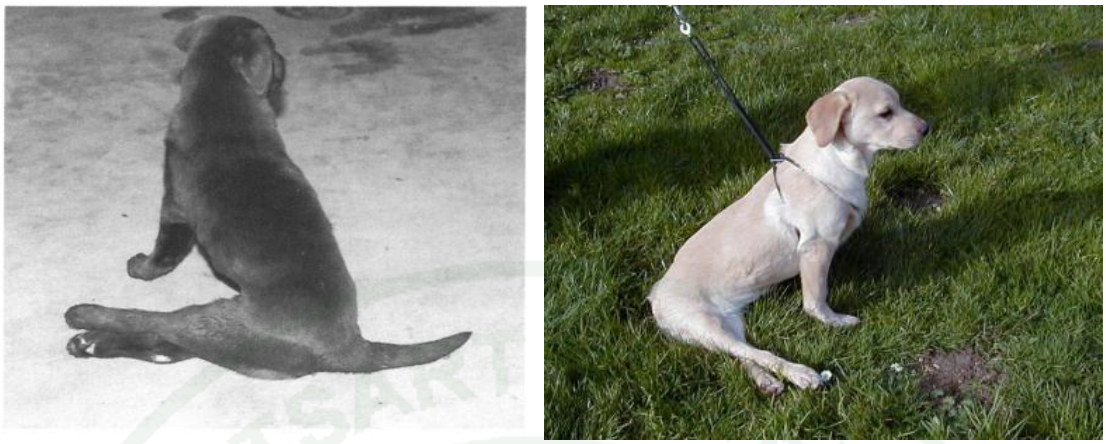


Figure 8 Dogs with hindlimb paralysis.

Source: Dubey and Lindsay (1993) ; Mark (2009)

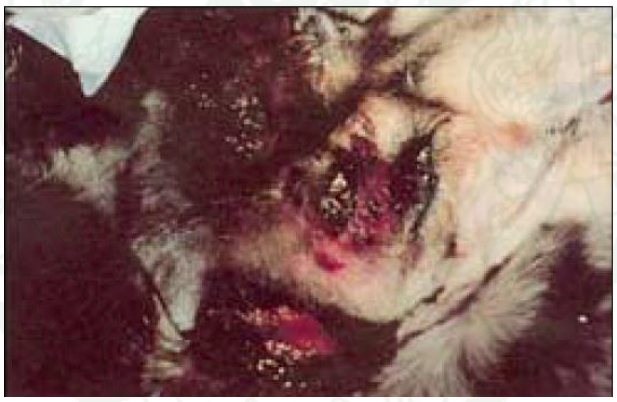


Figure 9 Hemorrhagic, necrotic, and pyogranulomatous dermatitis due to neosporosis in a 6-year-old dog.

Source: Barber (1998)

dogs of any age. A subclinically infected bitch can transmit the parasite to their fetuses, and successive litters can be born infected (Dubey *et al*., 1990b). However, breed predisposition and differential sex susceptibility to neosporosis in dogs is still unknown. Most reported cases have been found in Labrador retrievers, Boxers, Greyhounds, Golden retrievers, and Basset hounds (Dubey and Lindsay, 1996; Dubey, 2003).

Figure 10 Fifteen-week-old Labrador retriever puppy in left figure showing paraplegia and rigid hyperextension of both hindlimbs due to neosporosis. In the right, 11-month-old the same Labrador retriever, taken 6 months after completion of a 6-week course of clindamycin. Despite severe muscle wastage and joint deformities, the dog could walk for a short distance.

Source: Barber (1998)

3.2 Cattle

N. caninum is a major cause of abortion in both dairy and beef cattle worldwide (Dubey, 2003). Fetuses dying in utero between 3 and 8 months of gestation are usually expelled showing moderate autolysis, but fetuses dying before five months of gestation may be mummified and retained in the uterus for several months and those dying at an early stage of gestation may be reabsorbed (Anderson *et al*., 1991; Barr *et al*., 1991a; Gonzales *et al*., 1999; Morales *et al*., 2001b; Sager *et al*.,

2001; Moore *et al*., 2002). Mummification appears to be an important clinical finding in outbreaks of *N. caninum*-associated abortions in cattle (Thornton *et al*., 1991; Nietfeld *et al*., 1992). Fetuses may stillborn, born alive with clinical signs, or born without clinical sign, which persistently infected may exhibit neurologic symptom 1-2 weeks later. When the congenitally infected calves grow up, the neosporosis may drop or deteriorate, or become subclinical infection. In some cases, *N. caninum* may cause death fetuses and were normally found more abundant in early gestational fetuses than in late gestational fetuses. Tissue cysts were also more common in early gestational fetuses than in the late gestational fetuses (Anderson *et al*., 1991; Barr *et al*., 1991a).

Clinical signs, other than abortion, have rarely occurred in calves less than 1 month of age such as neurological signs, weak, unable to rise, and below average birthweight. The hind limbs or forelimbs, or both, may be flexed or hyperextended (Figure 11) and neurological examination may reveal ataxia, decreased patellar reflexes, and a loss of conscious proprioception. Exophthalmia or an asymmetrical appearance in the eyes has been reported and occasionally birth defects including scoliosis, hydrocephalus and a narrowing of the spinal cord can occur (Parish *et al*., 1987; O'Toole and Jeffrey, 1987; Barr *et al*., 1993; Dubey and de Lahunta, 1993; Dubey *et al*., 1998a).

Figure 11 A neosporosis calf with underweight, weak and unable to rise.

Source: Dubey and Lindsay (1993)

4. Epidemiology

4.1 Dairy and beef cattle

Seroprevalence of *N. caninum* infection in dairy and beef cattle which have been reported worldwide are summarized (Table 1).

Table 1 Reports of seroprevalence of *N. caninum* infections in cattle worldwide

Country	Location	Animals	Number tested	% positive	Test	References
United States	California	calves	278	30.6	ELISA	Pare *et al*., 1994
			127	53.5		
		cows	228	36.0		
			57	57.9		
	Georgia	cows	327	32.11	Western blot	Ortega *et al*., 2007
	Texas	cows	87	10.34	Western blot	Ortega *et al*., 2007
	Maryland	Neonates	107	17.9	IFAT	Dyer *et al*., 2000
		heifers and steers	223	26.2		
		mature heifers	218	39.07		
		milking cows	465	26.9		
Canada	New Brunswick	cows	900	25.5	ELISA	Haddad *et al*., 2005
	Nova Scotia		900	21.3		
	Ontario		930	8.2		
	Prince Edward Island		900	10.4		
Costa Rica	Poas	cows	3002	39.7	ELISA	Romero *et al*., 2002
Mexico	central plateau	cows	1003	56	ELISA	Morales *et al*., 2001a
Argentina	Humid Pampas	cows	1048	16.6	IFAT	Moore *et al*., 2002
		cattle	400	4.7		
Brazil	Bahia	cows	447	14.09	IFAT	Gondim *et al*., 1999

Table 1 (Continued)

Country	Region	Samples	Number tested	% positive	Test	References
	Rio Grande do Sul	cows	223	11.2	IFAT	Corbellini et al., 2002
	Parana State	cows	172	34.8	ELISA	Locatelli-Dittrich *et al*., 2001
Paraguay	different locations	cattle and cows	879	29.8	ELISA	Osawa *et al*., 2002
Denmark	-	cows	1561	22	ELISA and IFAT	Jensen *et al*., 1999
France	Normandy	cows	1924	5.6	ELISA	Ould-Amrouche *et al*., 1999
Sweden	middle Southern Sweden	cows	112	16	ELISA	Bartels *et al*., 2006
Germany	Rhineland-Palatinate	cows cattle	100 106	49 41	ELISA	Bartels *et al*., 2006
Spain	Galicia	cows cattle	291 372	63 46	ELISA	Bartels *et al*., 2006
The Netherlands	-	cows cattle	108 82	76 61	ELISA	Bartels *et al*., 2006
Italy	Potenza Padua	cattle and cows cows	1140	8.7 16	ELISA	Otranto *et al*., 2003
England	Near Liverpool	cows	4295	17.1	ELISA	Davison *et al*., 1999
Australia	New South Wales	cows	266	24 29	IFAT immunoblot	Atkinson *et al*., 2000
Japan	-	cows cattle	145 65	20 1.5	IFAT	Koiwai *et al*., 2005

Table 1 (Continued)

Country	Region	Samples	Number tested	% positive	Test	References
New Zealand	-	cows	1199		IFAT and ELISA	Reichel and Pfeiffer, 2002
Korea	many provinces	cattle	852	12.1	ELISA	Ahn *et al*., 2003
Vietnam	Ho Chi Minh City	cows	200	5.5	ELISA	Huong *et al*., 1998

The first report of *N. caninum* infections in Thailand was studied in 11 provinces of central Thailand, cattle was tested by IFAT and 6% was found positive (Suteeraparp *et al*., 1999). This was also the first report of *Neospora*-associated abortion in Southeast Asia. The first identification of *N. caninum* parasite in Thailand was demonstrated by collected aborted bovine tissues from two high abortion rate (7%) of dairy farms in Chonburi and Saraburi provinces and *N. caninum* were detected by immunohistochemical examination (Kyaw et al., 2003). Five out of 12 aborting cows (41.7%) were seropositive to *N. caninum* using the competitive ELISA (cELISA). Tachyzoites of *N. caninum* were also detected in the placenta of a seropositive aborting cow. Neither *N. caninum* tachyzoites nor cysts were found in other fetal tissues. Kyaw *et al*. (2004) also studied the seroprevalence of cows and dogs in dairy herds in Nakhon Pathom and individual and herd seroprevalence in cows were 5.5% and 34%, respectively. No significant relationships between the seropositivity and age of the cows were found. The seroprevalence of *N. caninum* in dogs was 1.2%. This was the first *N. caninum* seroprevalence reported in dogs in Thailand. However, herd infection and the presence of dogs in the farm were not related. In northeast Thailand, the seroprevalence of *N. caninum* infections of cow in Loei and Nong Bua Lamphu province were ranged from 37.5%-70% (Kashiwazaki

et al., 2001). Furthermore, antibody in bulk milk testing was tested to confirm *N. caninum*-infected herds using an iscom ELISA in northeast and north of Thailand and a total of 46% were positive (Chanlun *et al*., 2002). In 2008, Jittapalapong et al. reported the seroprevalence of *N. caninum* infections in dairy farms of northeast Thailand including Khon Kaen, Udorn Thani, and Sakon Nakhon province were 58.2%. Khon Kaen province had the highest infection of *N. caninum* (12.9%).

4.2 Water buffaloes

Although, these are not abundant like in the cattle, water buffaloes have become economically important animals. The studies of *N. caninum* infection in water buffaloes are summarized in Table 2.

Table 2 Reports of seroprevalence of *N. cani*num infections in water buffaloes worldwide

Country	Location	Animals	Number tested	Positive (%)	Test	References
Egypt		water buffaloes	75	68	MAT	Dubey *et al*.,1998b
Brazil	Ribeira Valley of São Paulo State	water buffaloes	222	64 53	IFAT NAT	Fujii *et al*., 2001
Argentina	Corrientes	water buffaloes	449	64	IFAT	Campero and Perez, 2007
Italy	Caserta	water buffaloes	1377	34.6	IFAT	Guarino *et al*., 2000
Vietnam	Ho Chi Minh City	beef water buffaloes	200	1.5	ELISA	Huong *et al*., 1998
India	Punjab		32	50	ELISA	Meenakshi *et al*.,2007
Iran	Ahvaz		181	37	ELISA	Hajikolaei *et al*., 2007
Philippines	Luzon		105	3.8	ELISA	Konnai *et al*., 2008
	Nueva Ecija		176	27.3	cELISA	Abes and Divina, 2008
Pakistan	Punjab		300	54.7	ELISA	Nasir *et al*., 2011

The first report of the seroprevalence of *N. caninum* infection in water buffaloes in Thailand was investigated by Wiengcharoen *et al*. (2010), who collected blood from 30 water buffaloes of a local farm in Chachoengsao province. The seroprevalence were 73.33% by cELISA. The high prevalence of *N. caninum*

infection indicated that *N.caninum* might be spread in that farm. Nam *et al*. (2012) also reported 4.5% of seropositive water buffaloes in the Northeast.

4.3 Dogs

In the epidemiological study of dairy farm, the presence of dogs in the farm was likely a risk factor for *N.caninum* infection in cattle (Dijkstra *et al*., 2002). Defecation by farm dogs on feeding alleys and stored grass or corn silage were reported more often among herds with an evidence of postnatal bovine infection (Dijkstra *et al*., 2002). The herds with the postnatal infection were observed, dogs that feed with bovine placenta, uterine discharge, and colostrum or milk were more often found than the control herds (Dijkstra *et al*., 2002). This suggested that infected cattle tissues may pose an infection risk to dogs more than by those of herds with no such evidence. Oocysts shedding by dogs are the key factor in the epidemiology of neosporosis. (Dubey *et al*., 2007). Seroprevalence of *N.caninum* infection of dog was reported worldwide and summarized in Table 3.

Table 3 Reports of seroprevalence of *N. cani*num infections in dogs worldwide

Country	Location	Animals	Number tested	Positive (%)	Test	Reference
Argentina		dogs from dairy farm dogs from beef farm dogs from urban area	125 35 160	48 54.2 26.2	IFAT	Basso *et al*., 2001a
Brazil	Parana	dogs from dairy farms	134	21.6	IFAT	de Souza *et al*., 2002
Italy	Bari Bari and Taranto	kennel farm farm dogs	144 162	14.6 26.5	Inhibition ELISA	Paradies *et al*., 2007
Korea		urban dogs dogs from dairy farm	289 51	8.3 21.6	IFAT	Kim *et al*., 2003
Mexico	Hidalgo	farm dogs city dogs	27 30	51 20	ELISA	Sanchez *et al*., 2003
Spain	Catalonia	different veterinary clinics	139	12.2	IFAT	Ortuno *et al*., 2002
Taiwan	Taichung	dogs from dairy farms	13	23.07	IFAT	Ooi *et al*., 2000

In Thailand, there were the reports of seroprevalence of *N.caninum* in dogs as 1.2% reported by Kyaw *et al*. (2004), 11.1% by Chanlun *et al*. (2007) and 4.4% by Arunvipat *et al*. (2012).

5. Risk factors

Risk factors of herds to acquire *N. caninum* infection and *N. caninum*-associated abortion are important for the development and implementation of means to control bovine neosporosis. There are many risk factors which have been examined at the herd or individual level with serostatus. The results of these studies were correlated with the sensitivity and specificity of serological technique. However, the detection of *Neospora* infection by using serological tests does not give information on the actual infection and the route of infection (Dubey *et al*., 2007).

5.1 The age

There were many reports to indicate the age of animals as a risk factor. The study in Germany, The Netherlands, Spain, and Sweden was significantly shown the association between age and seropositivity (Bartels *et al*., 2006). In Spain, the risk of being seropositive increased with age, while in Sweden, the situation was the opposite (Bartels *et al*., 2006). The risk of being seropositive might increase with age or gestation number in beef and dairy cattle (Dyer *et al*., 2000; Sanderson *et al*., 2000; Rinaldi *et al*., 2005), suggesting that horizontal transmission of *N. caninum* is of particular importance in some herds. In Spain, it was hypothesized that the age effect might be influenced by variations in the probability of horizontal transmission (e.g., by the risk of ingesting oocysts), by regional differences regarding replacement rate (influencing the time cattle may be exposed to horizontal transmission), and by management practices such as selective culling of seropositive animals (Bartels *et al*., 2006). A study of water buffaloes in Italy, the prevalence increased in relation to the age of subjects and most of the examined herds were found infected (Guarino *et al*., 2000). The higher seropositives in older animals suggested that the transplacental route might not be likely the common mode of transmission since buffaloes might get infected by consuming oocyst contaminated food or water. In UK, dairy herds were tested for *N. caninum*-specific antibodies and found that there was a significantly lower prevalence in 13-to 24-month-old cattle than 7-to 12-month-old cattle and older cattle (Davison *et al*., 1999). It was hypothesized some of the 13- to 24-month-old

animals (most likely heifers) were congenitally infected with *N. caninum* with seronegative. Recrudescence during gestation might have been caused an elevated seroprevalence in older age groups (Davison *et al*., 1999).

5.2 The presence of dogs

The presence of dogs in the farm was a risk factor for seropositivity to *N. caninum* in cattle (von Blumroder *et al*., 2006) since dogs have been known as the definitive hosts of *N. caninum* (Dubey *et al*., 2007). Farm dogs might be the potential risk to dairy cattle due to their defecation on feeding alleys and stored grass or corn silage (Dijkstra *et al*., 2002). Dogs fed or contaminated with bovine placenta, uterine discharge, and colostrum might pose an infection risk (Dijkstra *et al*., 2002) because dogs might get infected with *N. caninum*. In an experimental study, cattle placenta has been confirmed as an infection source for dogs (Dijkstra *et al*., 2001). Interestingly, feeding on aborted fetuses was not identified as a potential risk factor in herds with evidence of recent postnatal infection (Dijkstra *et al*., 2002), and no oocyst shedding was observed when aborted fetuses or brains of fetuses were experimentally fed to dogs (Bergeron *et al*., 2001). However, these results were most likely influenced by the stage of fetus autolysis killing the parasite within the host cells. Most *N. caninum* in aborted fetuses die within the host cells, and it is unlikely to find intact tachyzoites in such tissues (Dubey *et al*., 2006). Dogs have sheded oocysts after ingesting a variety of tissues, including nervous tissues, muscles, internal organs, and fetal membranes. However, dogs might not shed or shed only a few oocysts after being fed repeatedly with infectious tissues (Dijkstra *et al*., 2001; Schares *et al*., 2001; Gondim *et al*., 2005). Additionally, higher oocyst numbers were sheded by young dogs (10 to 14 weeks old) than older dogs (2 to 3 years old) (Gondim *et al*., 2005).

In Germany, the dog population and density was the potential risk factor for *N. caninum* infections in dairy herds (Schares *et al*., 2003; von Blumroder *et al*., 2006) since farms with two or more dogs had higher herd seropositivity than farms with one or no dogs, as presented in beef and dairy cattle in Italy (Otranto *et al*., 2003). For beef cattle, there are no evidences that dogs kept in the surroundings of

farms pose an infection risk (von Blumroder *et al*., 2006). A possible explanation is that on the less intensively managed beef farms, there is in general no close contact between the excretions of dogs and beef cattle (Sanderson *et al*., 2000; Barling *et al*., 2001; Otranto *et al*., 2003).

5.3 The contaminated oocyst in the environment

Oocysts contaminated in the pasture, fodder, and drinking water are considered as potential sources for postnatal infection of cattle. Therefore, it is important to know which feeding practices pose the infection risk. In the northwestern USA and Italy, grazing of cattle on rangeland in summer was likely to be a protective factor (Sanderson *et al*., 2000; Otranto *et al*., 2003). Because the oocyst related by definitive hosts which have free access to rangeland, may be too low to pose a significant threat and oocysts might not survive during the hot and dry months. However, information on the climatic conditions influenced *N. caninum* oocysts survival in the environment is inconclusive.

In France, the use of ponds rather than the use of a well or public water supply for drinking water was reported as the risk factor for *N. caninum* infection in dairy cattle (Ould-Amrouche *et al*., 1999).

5.4 Feeding colostrum or milk

In experimental study, six calves were inoculated with 10^7 *N. caninum* tachyzoites in colostrum and/or milk replacer. All calves developed antibody responses to *N. caninum* after ingestion of milk containing tachyzoites (Davison *et al*., 2001). However, cross-suckling of calves born to seronegative mothers on seropositive cows has not led to an infection (Davison *et al*., 2001). Because *N. caninum* DNA was found in bovine milk, these findings implicated the possibility of *N. caninum* transmission through the colostrum (Moskwa *et al*., 2007). Corbellini *et al*. (2006) suggested that cattle from farms that fed calves with colostrum pooled from multiple cows have a putative risk for seropositivity (Corbellini *et al*., 2006).

5.5 Herd size and cattle density

Otranto *et al.* (2003) reported the risk of being seropositive increased in larger herds with an increasing number of dogs in farm. In Germany, larger herds had an increased risk of being bulk milk positive since increasing herd size had an increasing chance of acquiring *N. caninum* infection by, for instance, the purchase of external replacement heifers (Schares *et al*., 2003). Moreover, hygienic controls to prevent dogs from feeding on placentas or other infectious materials are complicated within large herds more than in small herds (Schares *et al*., 2003).

In Texas, the high stocking density was identified as a potential risk factor for seropositivity (Barling *et al*., 2000, 2001). This was also confirmed by the evidence of the stocking density of beef cows in the Northwest, USA including Idaho, Montana, Oregon, Washington, and Wyoming (Sanderson *et al*., 2000). This effect was demonstrated that ranches with a high density of cattle are more likely to use supplemental feeding practices (Barling *et al*., 2000, 2001). Farm supplemental feed might attract rodents that were the potential prey for definitive hosts of *N. caninum*. Therefore, this could increase the risk of fecal contamination of definitive hosts, thus increasing the risk of postnatal infection (Barling *et al*., 2000).

In the South of Brazil, the increasing size of farmland decreased the seroprevalence in herds but this effect was not linked to the stocking density (Corbellini *et al*., 2006). In small farms, the dog was easily accessed to consume bovine carcasses, aborted fetuses, placenta, and uterine discharge compared to large farms.

6. Economic impact

Livestock development particularly in dairy cows has been hampered by low production including milk and slow growth rate due to many pathogens including *N. caninum*. After being recognized, *N. caninum* became a significant cause of bovine abortion throughout the world (Bjerkas *et al*., 1984). The parasite is passed from

mother to offspring and caused fetal death or weak calves. *N. caninum* is known to have a detrimental effect on bovine pregnancy and milk production due to its effect on reproductive failure in cattle, which make economic losses to farmers. The direct loss is due to abortion and indirect losses such as veteriny service expenses associated with a diagnosis, rebreeding, reducing milk yield, and a replacement for culled animals (Dubey *et al*., 2007).

Neosporosis is difficult to identify because there are no obvious clinical signs in adult cattle (Dubey *et al*., 2007). In California, *Neospora*-seropositive cows were culled 6.3 months earlier than Neospora-negative cows and had a 1.6 times greater risk of being culled, compared with seronegative (Thurmond and Hietala, 1996). Tiwari *et al*. (2005) reported that *N. caninum*-seropositive cows were culled at a rate 1.43 times higher than seronegative dairy cows in four Canadian provinces. Bartels *et al*. (2006) reported high seropositive cows increased the hazard for culling at 1.73-fold compared to negative and low seropositive.

In milk production effect, milk production of seropositive cows (55.2 pound/cow/day) was 2.5 pound/cow/day less than that for seronegative cows (57.7 pound/cow/day) in California. In Florida, exposure to *N. caninum* was caused a 3-4% decrease in milk production and represents a loss of $128/cow/lactation (Hernandez *et al*., 2001). Bartels *et al*. (2006) reported there was an effect of serostatus on milk production in the first year after an abortion epidemic. In the first 100 days in milk, cows with positive serostatus produced 0.59-0.72 kg. milk/day less.

Risks of abortion and stillbirth in seropositive cows were significantly greater than in seronegative cows. Risks of being culled for reproductive failure in seropositive cows were also significantly greater than in seronegative cows in central Alberta, Canada (Waldner *et al*., 1998). The analysis of 966 sera from aborted cows, demonstrated 18.9% positive from beef herds and also revealed that *N. caninum* was an important risk factor of reproductive losses in the extensively beef cattle farm in the Humid Pampas of Argentina (Moore *et al*., 2002).

Neosporosis on economic losses in the cattle industry, for example, in Australia and New Zealand, *N. caninum* caused significant losses to cattle producers, estimated to exceed AU$100 million per year (Reichel, 2000). In Switzerland, the losses due to *N. caninum* in the Swiss dairy cow population were estimated 9.7 million euros annually (Hasler *et al*., 2006). In Canada, losses were evaluated as $ 2,304 for a 50-cow dairy herd (Chi *et al*., 2002). Although, 76% of seropositive reference herds in The Netherlands had no economic losses, in the remaining 24% of herds increased the damages to 2,000 euro per year (Bartels *et al*., 2006). In a seroepidemiological study, an economic loss of $15.62 per calf has been shown by Barling *et al*. (2000). Furthermore, the costs were an average of 25 euros/ animal/ year following the abortion epidemic and including premature culling, prolonged calving interval and the age of first calving, milk production losses, treatment, and diagnosis (Bartels *et al*., 2006). Currently, there are no effective drug to treat neosporosis, so the culling of seropositive cattle is still the method to control the disease.

7. Diagnosis of *N. caninum* infection

The diagnosis of bovine Neosporosis is confirmed by the clinical signs, histological lesion of fetal tissue tachyzoites by immunohistochemistry, serological method, and molecular application.

7.1 Immunohistochemical examination (IHC)

Immunohistochemistry, the first test established to identify the parasite (Zhai *et al*., 2007), is frequently used for demonstration of *N.caninum* infection. Moreover, there were no cross-reaction with the closely related *T. gondii* or other extra-intestinal coccidian (Lindsay and Dubey, 1989). IHC staining is a reliable technique that demonstrated *N. caninum* in many organs including fetus and brain. Tissue cysts or trachyzoites can also be frequently found in lung, kidney and skeleton muscle (Figure 12) (Zhai *et al*., 2007). Cross-reactivity of *N. caninum* antibodies to related apicomplexans such as *T. gondii*, and *Sarcocystis spp*. can be found with less

concern because these protozoans are rarely associated with abortion in cattle (Anderson *et al*., 1991; Canada *et al*., 2002). One of the advantages is due to its high specificity, but some false positives in tissue sections of *T. gondii* infected animals has been reported (van Maanen *et al*., 2004). The disadvantage of IHC depends on a large extent on the number of sections made and the time spent on microscopic examination (Wouda *et al*., 1997).

7.2 Polymerase chain reaction (PCR)

PCR technique is an important diagnostic tool for a detection of *N. caninum* in aborted fetuses (Gottstein *et al*., 1998; Baszler *et al*., 1999; Sager *et al*., 2001; Pereira-Bueno *et al*., 2003; van Maanen *et al*., 2004; Medina *et al*., 2006). Generally PCR has the higher sensitivity and specificity than IHC methods (van Maanen *et al*. 2004) since PCR can amplify small amounts of *N. caninum* DNA. Most PCR protocols are used to detect *N. caninum* DNA in the tissues of aborted fetuses or other intermediate hosts. Moreover, amniotic fluid (Ho *et al*., 1997), cerebrospinal fluid (Peters *et al*., 2000; Buxton *et al*., 2001; Schatzberg *et al*., 2003), and feces of dog or coyote (Basso *et al*., 2001b; Hill *et al*., 2001; Slapeta *et al*., 2002; McGarry *et al*., 2003; Gondim *et al*., 2004b; Schares *et al*., 2005) have been detected by PCR for the presence of *N. caninum* DNA. However, the attempts to detect *N. caninum* DNA in blood of naturally infected cattle were not successful (Guy *et al*., 2001). Recently, Ferre *et al*. (2005) and Okeoma *et al*. (2004a) reported that there was the possibility to identify *N. caninum* DNA in the blood of chronically infected cattle (Figure 13) (Ferre *et al*., 2005; Okeoma *et al*., 2004a). PCR can also detect *N. caninum* DNA in the milk of lactating cows (Moskwa *et al*., 2003) and in the semen of bulls (Ortega-Mora *et al*., 2003; Caetano-da-Silva *et al*., 2004; Ferre *et al*., 2005). PCR was developed not only to detect but also to quantify *N. caninum* DNA in samples.

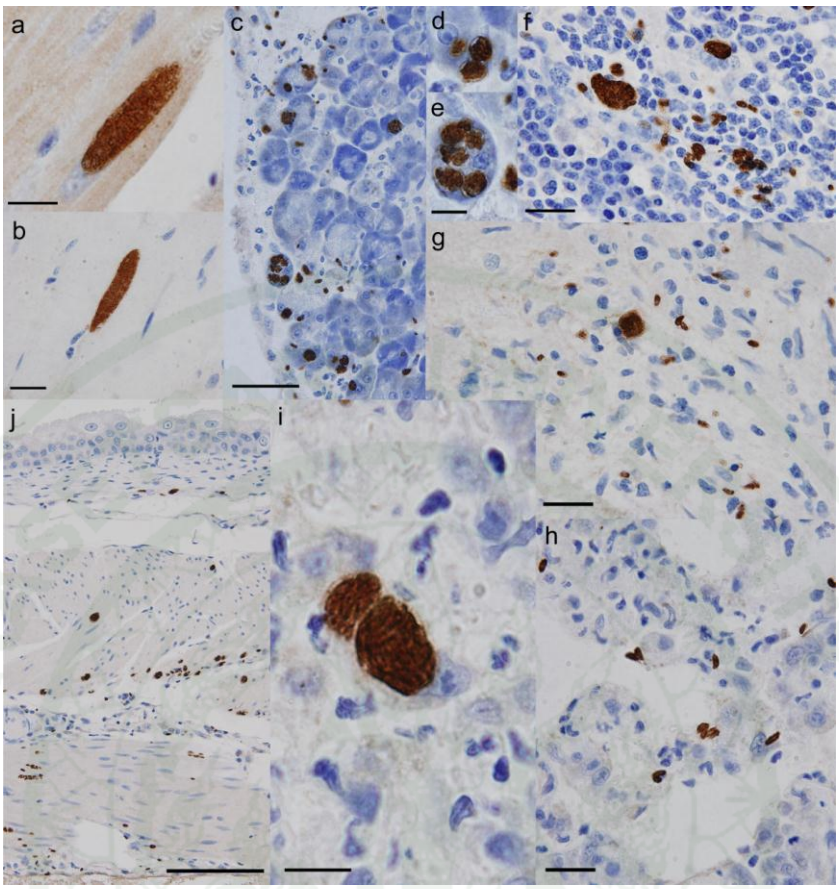


Figure 12 Immunohistochemical detection of *N. caninum* developmental stages in fat-tailed dunnart: Positive IHC staining with anti-Neospora antibodies: staining of elongated tissues cysts of *N. caninum* in cardiac (a) and skeletal muscle (b); pancreas containing a large number of free zoites (c) and multiple round cysts within a single acinar cell (d, e); lymph node (f), brain (g) and lung (h) with scattered developmental stages including free zoites; lung tissue with a large round cyst filled with zoites within a large mononuclear cell likely to be a pulmonary macrophage (i); and a cross-section of the urinary bladder wall with *N. caninum* stages apparent in all layers of the detrusor muscle (j). Tissues from animals inoculated 10^5 *N. caninum* tachyzoites. Bars: A, B, F, G, H = 20 μm; C = 50 μm; D+E, I = 10 μm, and J = 100 μm.

Source: King *et al*. (2011)

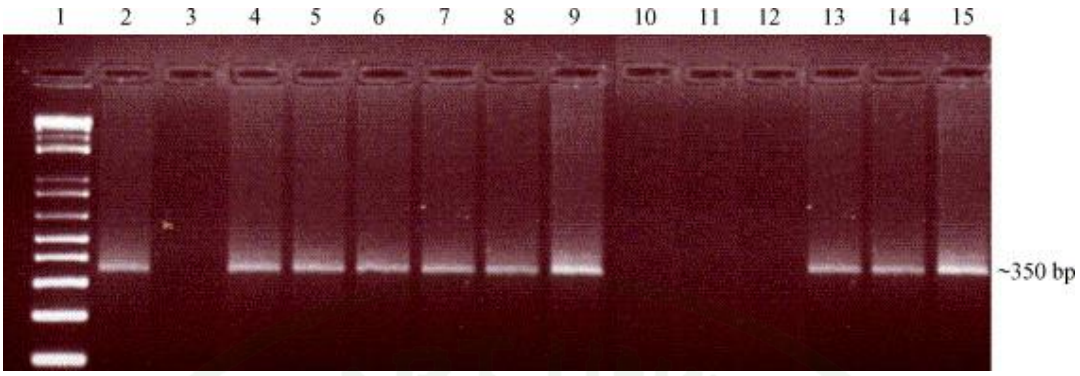


Figure 13 PCR product of Nc-5 fragment amplified with primer pair Np21+ and Np6+. Lane 1: 1 kb+ ladder, lanes 2 and 3: positive and negative controls, respectively, lanes 4–9: products from sero-positive heifers after abortion, lanes 10–12: products from sero-negative pregnant heifers, lanes 13–15: products from sero-positive pregnant heifers.

Source: Okeoma *et al*. (2004a)

7.3 Indirect Fluorescent Antibody Test (IFAT)

The Indirect Fluorescent Antibody Test (IFAT) is the first serological test to demonstrate antibody to *N. caninum* (Dubey *et al*., 1988a) and was widely used to detect *N. caninum* infection in dogs and cattle (Conrad *et al*., 1993, Otter *et al*., 1997, Atkinson *et al*., 2000). IFAT has been used to detect antibodies from a large number of animal species including dogs, fox, cats, cattle, sheep, goat, water buffaloes, horses, rodents and primates (Zhai *et al*., 2007). IFAT has been used as the reference test (gold standard) (Bjorkman and Uggla, 1999). IFAT is based on the principle of affixing intact tachyzoites to microscopic slides which are incubated with the diluted test serum and in a second step with fluorescein-labelled antibodies directed against immunoglobulins of the animal species under investigation (Figure 14). Briefly, attaching whole tachyzoites on microscopic slides, then incubated with the diluted sample serum, and fluorescence-labelled antibodies directed againt immunoglobulin of the animal species under investigation. The IFAT result is considered positive when unbroken tachyzoite membrane is shown bright fluorescence (Figure 15) which

were found with tested moderate or high-titre sera (Pare *et al*., 1995). The low-titre sera are tested and apical with unbright green fluorescence occurs so that this might also occur as a result of cross-reactivity with *T. gondii* (Zhai *et al*., 2007). The cut-off titre in IFAT differs between laboratories from 1:100 to over 1:640 for adult bovines and from 1:16 to 1:80 for foetal serology (Bjorkman and Uggla 1999, Alvarez- Garcia *et al*., 2003). Performances of this test require training and experience and the result depends on the subjectivity of the reader. It is imperative that the optimal dilution of the fluorescein-labelled secondary antibody (the conjugate) is required to optimize with known positive and negative control sera along with the particular microscope used.

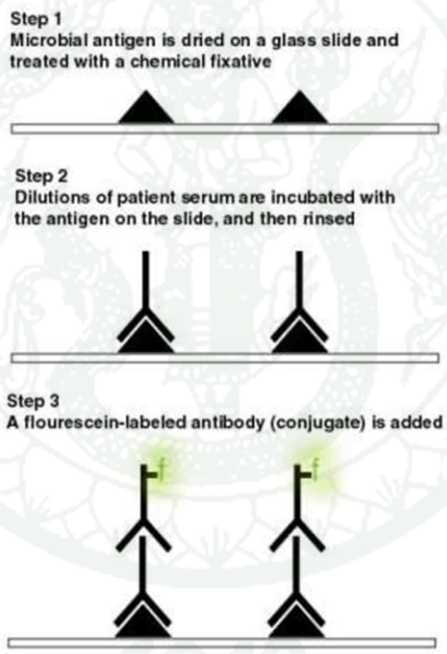


Figure 14 A concerp of Indirect Fluorescent Antibody Test (IFAT). Step 1.Microbial antigen is dried on a glass slide and treated with a chemical fixative Step 2.Dilutions of patient serum are incubated with the antigen on the slide, and then rinsed Step 3.A fluorescein-labeled antibody (conjugate) is added Step 4.The slides are rinsed and dried, and then read under a fluorescence microscope.

Source: Texas Department of State Health Services (2010)

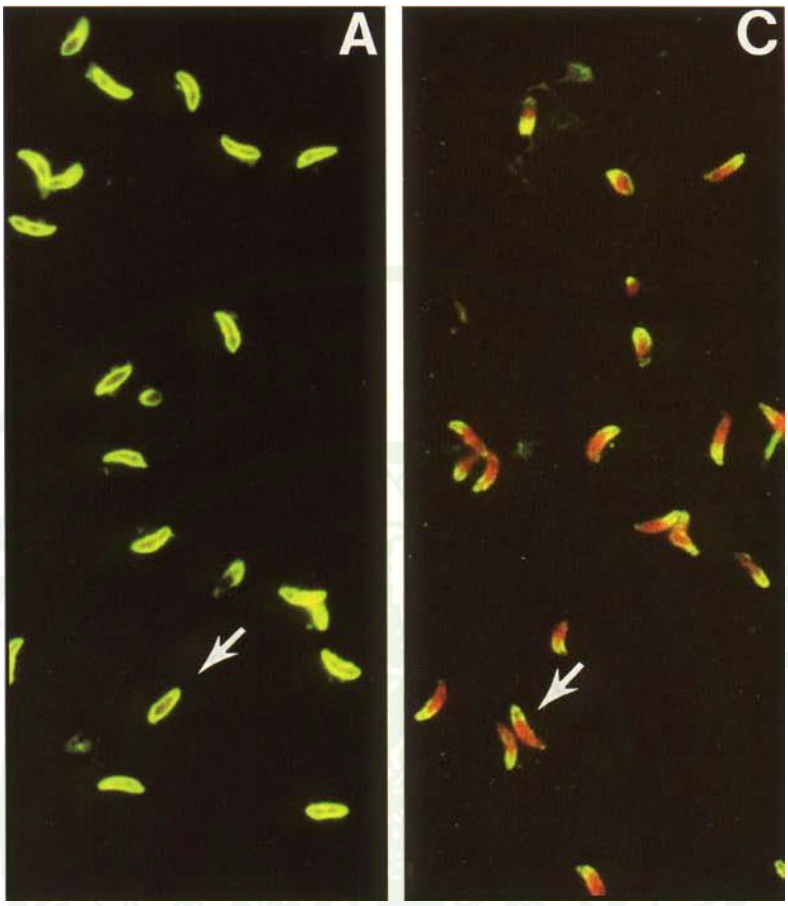


Figure 15 Positive IFAT result and negative IFAT result. A. Positive IFAT result with tachyzoite presenting nonapical fluorescence (arrow). C. Negative titer with tachyzoite presenting apical (nonspecific) fluorescence (arrow).

Source: Pare *et al*. (1995)

Because intact tachyzoites are used as antigen in the IFAT, the test mainly detects antibodies directed to antigens present on the cell surface of the parasite. With apicomplexan species, such antigens are considered more specific than intracellular components (Bjorkman *et al*., 1994). Infection studies in different hosts have shown that IFAT shows very little cross-reactivity with other coccidian parasites (Trees *et al*., 1993). This is particularly important in relation to *T. gondii*. For this reason, IFAT is often used as a referent test for *N. caninum* antibodies with which other assays are compared.

7.4 Enzyme-linked immunosorbent assays (ELISA)

Diagnosis of *N. caninum* infection can be confirmed by IHC or PCR (Dubey and Lindsay, 1996). However, diagnosis by direct detections (IHC or PCR) are often difficult because the low number of parasites in tissues and not available for post-mortem. Serological tests which can identify *N. caninum*-infected live animals provide valuable tools for diagnosis and epidemiological surveys as well as for experimental investigations. Due to the inconveniences inherent in the IFAT, there has been increasing interest in the development of ELISAs for the sero-diagnosis of neosporosis which enables rapid processing of large numbers of samples.

Indirect ELISA and competitive ELISA have been developed to diagnose neosporosis in cattle. Different antigen preparations have also been used, but the most commonly used is an indirect ELISA based on soluble tachyzoite antigens. ELISA has the advantage that the reaction is showed objectively and the assay can be easily performed. Therefore, ELISA is a practical technique for processing of a large number of samples. Addittionally, milk can be also tested by using this technique.

Different ELISA formats such as indirect ELISA and competitive-inhibition (CI)-ELISA have been developed. Different antigen preparations have also been used, but the most commonly used is an indirect ELISA based on soluble tachyzoite antigens. Both serum and milk can be tested using this technique.

8. The buffalo

A review of available literature indicates the existence of several types and varieties of buffaloes, mostly in Asia, Africa and some European countries. Taxonomy classifies buffaloes as follows (Czerniawska-Piatkowska *et al*., 2010) (Integrated Taxonomic Information System):

Kingdom : Animalia (animals)

Phylum : Chordata (chordates)

Subphylum : Vertebrata (vertebrates)

Class : Mammalia (mammals)
Order : Artiodactyla (artiodactyls)
Family : Bovidae (bovids)
Subfamily : Bovinae
Genus : Bubalus (buffalo)
Species : Bubalus bubalis– Water buffalo
Bubalus depressicornis
Bubalus mephistopheles
Bubalus mindorensis
Bubalus quarlesi
Genus : Syncerus
Species : Syncerus caffer– African buffalo

The water buffalo or domestic Asian water buffalo (*Bubalus bubalis*) is frequently used as livestock in Asia, and also widely in South America, southern Europe, northern Africa and elsewhere. In 2010, FAO estimated that there were approximately 194 million water buffalo in the world and 97% of them (approximately 188 million animals) were in Asia (FAO, 2010). Water buffalo have been domesticated for more than 5,000 years. They have buttressed humanity's survival with their meat, horns, hides, milk, butterfat, and power, plowing and transporting people and crops.

The domesticated water buffalo is divided into two types such as river and swamp buffalo. River buffaloes can be found mainly in Egypt and in some European countries (e.g. Italy, Germany, Bulgaria and England). The natural behaviour of these animals is wallowing in water, especially on hot days. This variety is suitable for dairy use. The genome of river buffalo includes 50 paired chromosomes (2n = 50). In karyotype image, one can see morphological types of chromosomes, four of which are metacentric, six are submetacentric and forty are acrocentric (Kumar *et al*., 2007). River buffalo are usually black with curled horns.

Swamp buffaloes are mostly found in India, Bangladesh, and China. Because of their low milk productivity (1–2 kg. / day), they are kept for meat or as work stock. This buffalo prefers bathing in stagnant waters and mud. These buffaloes have 48 pairs of chromosomes (2n = 48). During mitotic metaphase one can easily notice six metacentric chromosomes, four submetacentric and 38 acrocentric (Kumar *et al*., 2007). These two types of buffaloes can be mated to produce offspring with 49 pairs of chromosomes (2n = 49). The acrocentric chromosome 24 remains unpaired. Mixed breed buffaloes were sometimes found reproductive problems such as males might be infertile, and females might have delayed calving periods (Czerniawska-Piatkowska *et al*., 2010). Swamp buffalo is black or white or both, with long, gently curved, swept-back horns.

Figure 16 The pictures of river buffalo (left) and swamp buffalo (right).

Source: International Buffalo Information Centre (2009)

The major role of buffalo in Thailand

The swamp buffalo is very important for conventional agricultural practice in Thailand. The buffalo was considered a good worker because no comparable animals or machines could work as well as the buffalo. Because it has big feet and is good at pulling with slow walking steps, Thai farmers use them to pull rice-digging

instruments for preparing the land to grow rice. The buffalo was also used as a transportation animal in the rural area which has no road. Because of its long horn that sometimes becomes as long as 3 meters across both horns, the buffalo was also used in war. However, most of today's buffalo have short horns.

Thai swamp buffaloes were used as draft aniaml up to 14 years old without problems. That is very long work life compared to other animals. On average, the buffalo works 5 hours a day and in one year, a buffalo can help to grow 9.7 to 13.4 Rai of rice (1 Rai in Thai is equivalent to 1600 m^2). Therefore, the buffalo is used 122 days a year. In the past, most Thai farmers grew only one rice crop per year. At present, Thai farmers can have 3 rice crops each year. Eventually, the buffaloes will be sent to the slaughterhouse for meat, and hides. A swamp buffalo with 592 kg. average live weight yields 277 kg. carcass and 215 kg. meat (Nanda and Nakao, 2003). A buffalo will contain 40.8 to 46.4% of carcass (meat and bone) based on live weight (Faarungsang, 2003). Buffalo carcass quality is inferior compared to cattle i.e. less in lean cut percentage, less in dressing percentage, higher in fat percentage, and higher in bone percentage. Buffaloes grow faster than cattle due to their better digestibility (Nanda and Nakao, 2003). The cost of fattening per kg. bodyweight is therefore much lower in buffalo than cattle (Chantalakhana, 2001). Buffalo meat is lean, tasty and often undistinguishable from beef. It contains lower saturated fat than beef and pork, which is a good dietary value. Buffalo meat contains 40% less cholesterol, 55% less calories, 11% more protein and 10% more minerals in comparison to bovine meat so is, therefore, healthier.

For more than 5000 years, buffalo have been used as a draft animal in agriculture. Buffaloes are widely used to plow or level land, plant crops, puddle rice fields, cultivate crops, pump water, haul carts and shallow draft boats, carry people, thresh grain, press sugar cane, haul logs, and much more. They are particularly suited to work on wet fields with a strong body, broad hooves, flexible pastern and fetlock joints. They can stay longer than mules or oxen while puddling fields for rice plantation. In high temperature summer, the buffalo prefer to keep its body in mud rather than stay in tree shade, like cattle not. Their wide, flattened hooves enable them

to pull a plough through muddy rice paddies where oxen can get bogged down (Nanda and Nakao, 2003). Termed as the ‘living tractor’, swamp buffalo provide 20–30% of farm power in rice growing in south China, Thailand, Indonesia, Malaysia, and the Philippines (Nanda and Nakao, 2003).

The pasture quality in Thailand is poor and barren but the buffalo is well adapted to this condition. Buffaloes can eat a variety of plant more than of cattle. The weeds in the swamp areas along the roads are a good source of food for buffalo. Therefore, the buffalo is a weed controller and an ecological maintenance animal. The buffalo has produced a lot of fertilizer that can help fertile the land. Dung, a valuable by-product of buffalo farming, is used as fertilizer and as fuel for cooking, heating, and making biogas (Nanda and Nakao, 2003). Dung manure is an excellent source of nutrients for crops. Normally, dung is heaped to mature for a few months before fill into field soil to enhance soil fertility. Buffalo dung is extensively used as fuel for cooking in India, Pakistan, and Bangladesh (Nanda and Nakao, 2003). Biogas production from dung would reduce the energy deficit in Bangladesh by 15% (Nanda and Nakao, 2003). In Thailand, dung is also used as a construction material (Chantalakhana, 2001).

MATERIALS AND METHODS

1. Study area

Six provinces with the highest water buffalo densities in northeast Thailand (DLD, 2010) (Table 4) were chosen for this study: Ubon Ratchathani, Surin, Buri Ram, Si Sa Ket, Sakon Nakhon and Roi Et (Table 5). The areas sampled primarily consisted of hilly terrain with small mountainous or highland areas, and were geographically divided into the Sakon Nakhon and Khorat basins. The northern part (Sakon Nakhon basin) includes Sakon Nakhon and Roi Et provinces, and the southern part (Khorat basin) contains Ubon Ratchathani, Surin, Buri Ram and Si Sa Ket provinces (Figure 17). Rivers, streams and many ponds were found throughout the study area. The climate is tropical with hot summers, rainy seasons and dry cool winters with some rain. Small pasture areas are dispersed throughout the region, mainly for cultivation of rice, and water buffaloes mainly graze in public pastures and in cultivation areas after harvest. Agriculture is the major sector of the local economy, with rice as the main crop.

Table 4 Buffaloes population in northeastern provinces, Thailand

Provinces	Number of buffaloes
Ubon Rachathani	121,169
Surin	105,533
Buri Ram	89,226
Si Sa Ket	87,999
Sakon Nakhon	78,105
Nakorn Phanom	60,188
Udon Thani	53,481
Roi Et	50,118

Source: DLD (2010)

Table 5 Buffaloes sample collection in northeastern provinces, Thailand

Provinces	Sample collection
Ubon Rachathani	138
Surin	73
Buri Ram	70
Si Sa Ket	61
Sakon Nakhon	205
Roi Et	81
Total	**628**

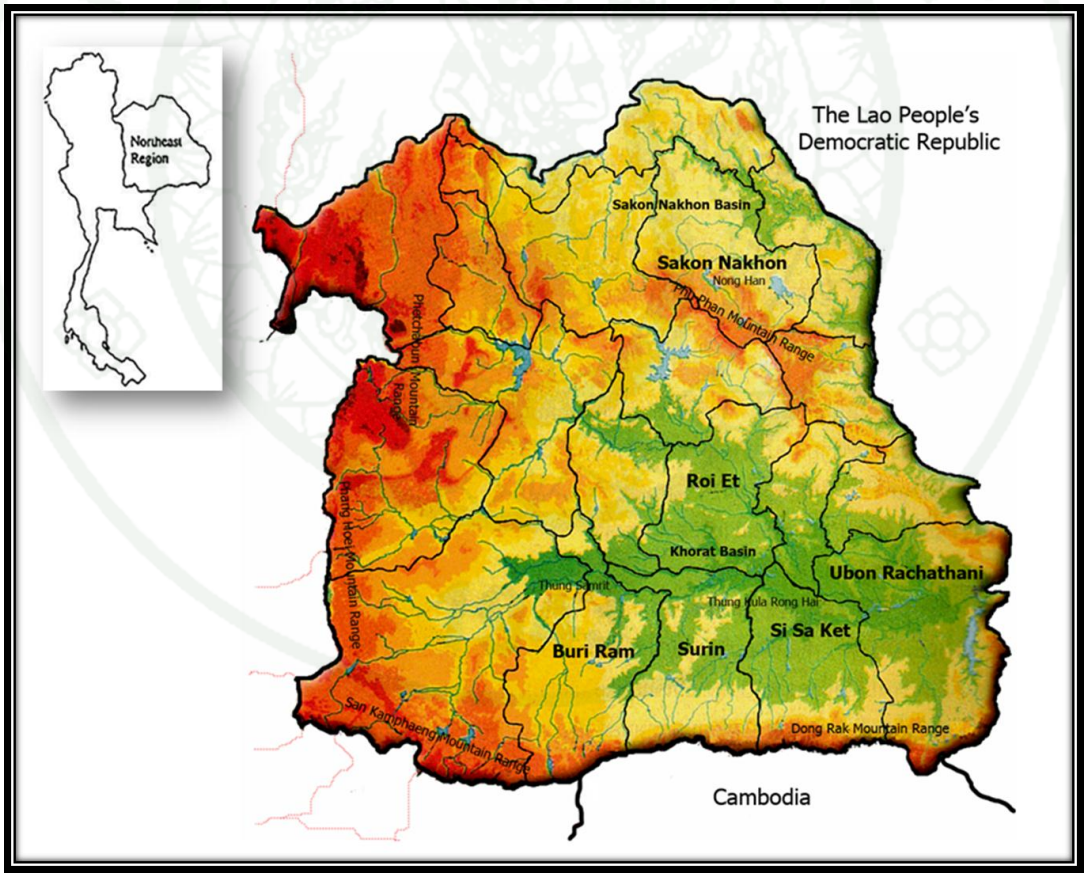


Figure 17 Map of northeastern provinces for sample collection, including Ubon Ratchathani, Surin, Buri Ram, Si Sa Ket, Sakon Nakhon, and Roi Et.

2. Sample size and distribution

Prior surveys for anti-*N. caninum* antibodies among cattle and water buffalo in Thailand reported seroprevalence of 4.5 to 73.3% with an average of 22.1% (Chanlun *et al*., 2007; Dubey *et al*., 2007; Nam *et al*., 2012; Wiengcharoen *et al*., 2012; Wiengcharoen *et al.*, 2010). Using this average to estimate the prevalence (p) of *N. caninum* among water buffalo in Thailand, a 95% confidence level (t) and 5% margin of error (m), the minimal sample size (n) of 69 was calculated based on the equation $n = t^2 \times p(1-p)/m^2$ (Padungtod, 2007). Farms selected for sample collection were distributed throughout the study area to avoid geographic clustering. The numbers of buffalo per farm is dependent on plantation area, because in Thailand buffaloes are conventionally used as draft animals for rice cultivation with a free range grazing system (Figure 18). On average, there are 3 to 5 buffalos/farm in northeast Thailand (DLD, 2010), thus buffalo herd sizes sampled in this study were divided into two groups of > 5 and ≤ 5 buffalo per farm.

3. Animal and blood samples

A total of 628 water buffalo samples were randomly collected from 288 farms of northeastern area of Thailand based on the buffalo population by probability proportional to size sampling (PPS) (Padungtod, 2007). Blood was collected from the coccygeal or jugular veins. For sera separation, blood was collected in sterile tubes without anticoagulant and centrifuged at 1448 G for 10 minutes, serum fractions were stored at -20°C until analysis (Figure 19). The animals sampled were divided into four age groups: $\leq$ 2.9 years (161 animals), 3 to 4.9 years (217 animals), 5 to 9.9 years (194 animals) and >10 years (56 animals) of age. Equal numbers of blood samples were collected from each age group present on the farm when possible.

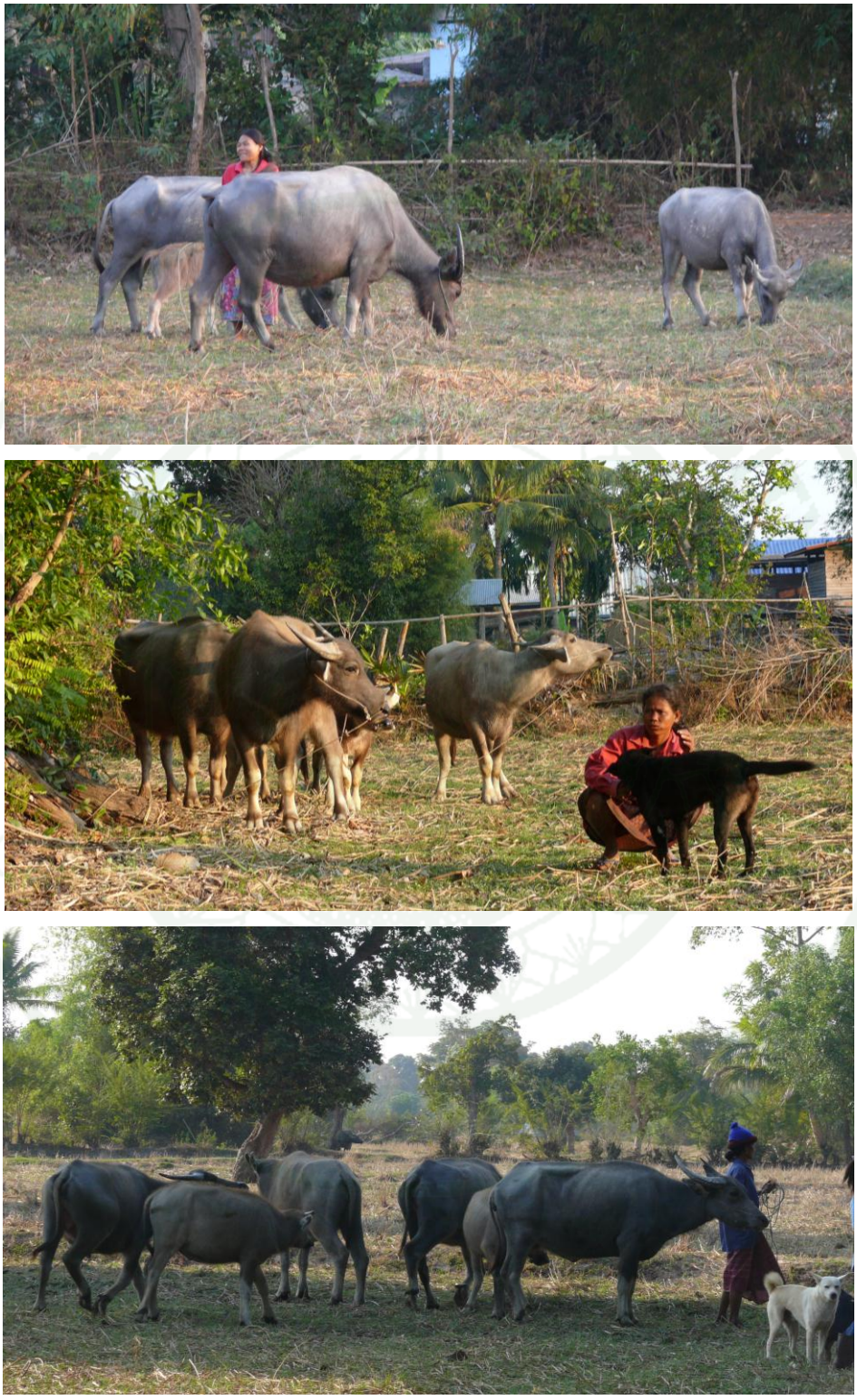


Figure 18 Buffalo husbandry and the presentation of dog in farm in northeast Thailand.

Figure 19 Blood sample collection from buffaloes in northeastern provinces, Thailand.

4. Factors associated with *N. caninum*

Factors including herd size, sex, and age of animals associated with *N. caninum* infection were analyzed by questionnaires from animal owners.

The other factor associated such as geographical areas was also analyzed. The northeast Thailand was divided into 2 parts such as the northern part and the southern part based on Sakon Nakhon basin and Khorat basin, respectively.

5. Culture of *N. caninum*

N. caninum tachyzoites (NC-1 strain) were maintained in African green monkey kidney (Vero) cells (Figure 20) and cultured in the minimum essential medium (MEM, Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine and penicillin-streptomycin at 37°C in 5% CO_2 air environment.

6. IFAT slide preparing

For the purification of *N. caninum*, adherent infected host cells were scraped from flasks and disrupted by passage three times each through 25 and 27-gauge needles. The parasites were then filtered through a 5.0 μm pore filter (Millipore, USA), and filtrate was centrifuged at 1,448 G at 4°C for 5 minutes, washed with 10 ml of PBS, and centrifuged again. Pelleted parasites were counted and diluted to 1×10^4 tachyzoites/mL before dispensing 10 μl of *N. caninum* tachyzoite suspension into each 4-mm well of teflon-coated antigen slides (Cel-Line Associates, Newfield, NJ) (Figure 21). Slides were then air dried at room temperature, fixed with acetone for 30 minutes, and stored at -20°C.

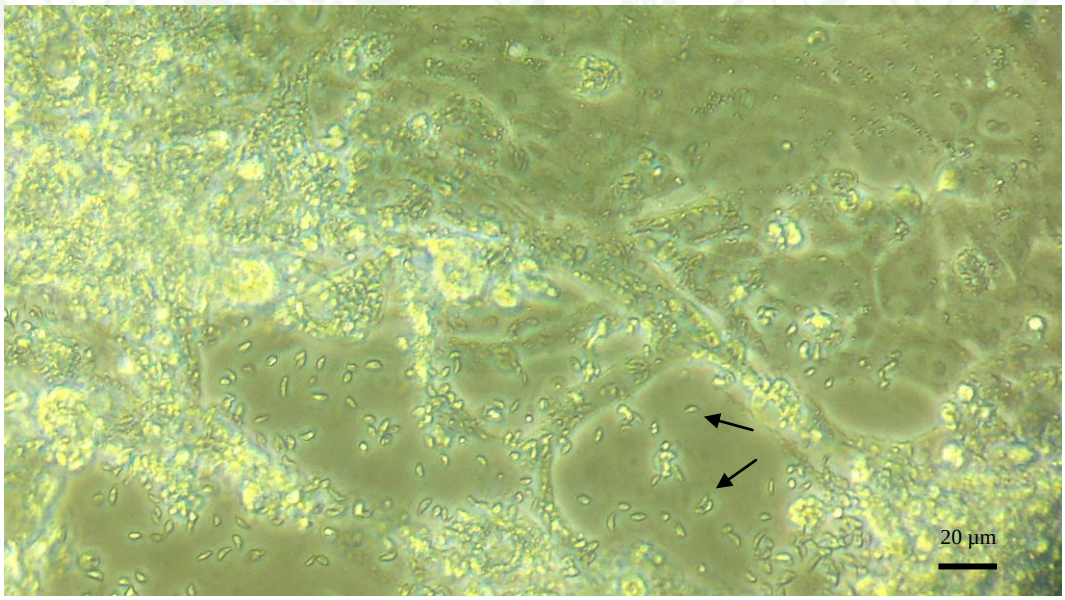


Figure 20 *N. caninum* crescent shape tachyzoites (arrow) in infected cells.

5. Diagnosis technique

The indirect fluorescent antibody test (IFAT)

IFAT was used to examine the reactivities of the sera to the isolated parasite and other antigens. Samples and control sera were diluted to 1:100 in PBS with 4% bovine serum albumin, placed onto 4-mm well of coated antigen slides, and incubated at 37 °C for 30 minutes. These slides were then washed three times with PBS, and incubated with 10 μL/well of caprine anti-bovine IgG1,2 FITC conjugate (VMRD, Pullman, WA, USA) and then incubated for 1 hour at 37°C. After incubation with secondary antibody conjugate, these slides were again washed three times with PBS, covered with cover slips and examined with a fluorescence microscope. *Neospora*-positive control sera were obtained from cows that were experimentally infected with NC-1 tachyzoites, and negative control sera were obtained from seronegative cows that were inoculated with uninfected Vero cells.

Figure 21 *N. caninum* tachyzoite suspensions were dispensed into each 4-mm well of teflon-coated antigen slides

6. Statistical analysis

Characteristics of individual water buffaloes sampled and information about the different farms were analyzed in relation to seroreactivity to identify putative risk factors associated with water buffalo exposure to *N. caninum*. Potential risk factors tested included province, geographic area (*i.e.*, basin), herd size, host sex and host age, which were analyzed for each province with the Chi-square (χ^2) test in Number

Cruncher Statistical System (NCSS) version 2000 (Kaysville, UT) programs. All of these factors were also assessed for potential association with exposure to *N. caninum* at the 95% confidence interval with WinEpiscope software version 2.0 (Thrusfield et al., 2001).

RESULTS AND DISCUSSION

Results

1. IFAT results

The positive result were shown as bright green fluorescence with unbroken membrane of tachyzoites compared to the negative results as unbright green fluorescence (Figure 22).

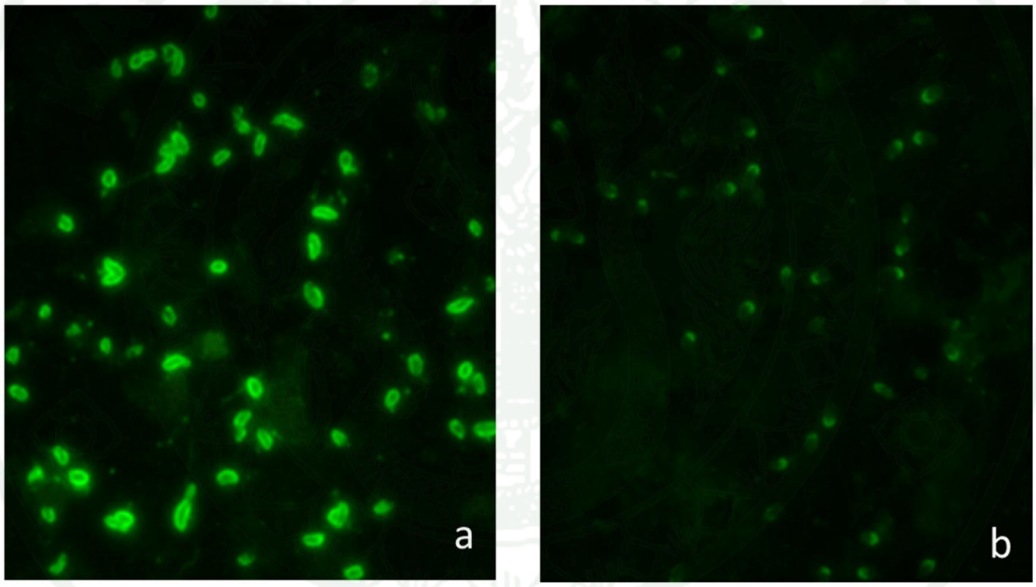


Figure 22 *N. caninum* tachyzoites with positive (a) and negative (b) by IFAT under fluorescence microscope (40x)

2. Seroprevalence of *N. caninum* infection

Individual host and herd-level seroprevalence values for anti-*N. caninum*-positive samples from water buffaloes in northeast Thailand are shown in Table 8. The *N. caninum* seroprevalence among individual water buffaloes ranged from 3.9% to 16.7% in the six provinces sampled in northeast Thailand, with an overall seroprevalence of 9.1% among individual hosts. The highest host seroprevalence was

among samples from Ubon Ratchathani province, followed by Si Sa Ket (14.8%, 9/61) and Surin province (9.6%, 7/73). Herd seroprevalence was 16.7% (48/288) among all of the farms sampled, and Si Sa Ket province had the highest herd seroprevalence (36.4%, 8/23) followed by Ubon Ratchathani (30.5%, 18/59) and Buri Ram province (22.7%, 5/22).

Among potential risk factors evaluated, seroprevalence was associated with host age, herd size and basin region of northeast Thailand (Table 6). A statistically significant association was not observed between seroprevalence and host sex. Farms with > 5 animals had higher seroprevalence (30 %) than those with ≤ 5 (16.2%), but these values were not statistically significant (p = 0.2). The highest seroprevalence was measured among water buffaloes > 10 years old (16.1%), followed by 5-10 years (13.4%), 3-5 years (9.2%) and < 3 years (1.2%) of age. Interestingly, a significantly higher seroprevalence was measured from the Khorat basin than from the Sakon Nakhon basin. The difference of prevalence was varied by host age, herd size, gender and the region of northeast Thailand shown in Table 6.

Table 6 Detection of antibodies to *N. caninum* from water buffaloes in northeast Thailand.

Parameter	IFAT+/Total (%)	Statistical parameters	Odd ratio 95% CI
Individual prevalence			
Province		χ^2= 20.4, df = 5, p = 0.001	
Ubon Ratchathani	23/138 (16.7%)		
Si Sa Ket	9/61 (14.8%)		
Surin	7/73 (9.6%)		
Buri Ram	6/70 (8.6%)		
Roi Et	4/81 (4.9%)		
Sakon Nakhon	8/205 (3.9%)		
Total	**57/628 (9.1%)**		
Basin		χ^2= 15.2, df = 1, p = 0.0001	
Sakon Nakhon	12/286 (4.2%)		0.2-0.5
Khorat	45/342 (13.2%)		1.9-6.5
Age group		χ^2= 19.7, df = 3, p = 0.0002	
< 3 years	2/161 (1.2%)		
3-5 years	20/217 (9.2%)		
5-10 years	26/194 (13.4%)		
> 10 years	9/56 (16.1%)		
Sex		χ^2= 2.83, df = 1, p = 0.09	
Male	4/91 (4.4%)		0.2-1.2
Female	53/537 (9.9%)		0.9-6.6
Herd prevalence			
Province		χ^2= 24.9, df = 5, p = 0.001	
Si Sa Ket	8/23 (36.4%)		
Ubon Ratchathani	18/59 (30.5%)		
Buri Ram	5/22 (22.7%)		
Surin	6/28 (21.4%)		
Sakon Nakhon	7/98 (7.1%)		
Roi Et	4/58 (6.9%)		
Total	**48/288 (16.7%)**		
House holding number		χ^2= 1.3, df = 1, p = 0.2	
≤ 5 animals	45/278 (16.2%)		0.1-1.8
> 5 animals	3/10 (30.0%)		0.6-8.6

Discussions

The results of this investigation indicated that water buffaloes are exposed to *N. caninum* in northeast Thailand, with an overall seroprevalence of 9.1% among individual hosts and an overall herd prevalence of 16.7%. To the best of our knowledge, this is the first report of risk factors associated with water buffalo exposure to *N. caninum* or of herd-level seroprevalence of the parasite among water buffaloes in Asia. Putative risk factors for exposure to the parasite included province, geographic area and host age. All of the provinces with the highest individual and herd-level seroprevalence were located in the Khorat Basin of northeast Thailand, which was over three-fold higher seroprevalence than the Sakon Nakhon Basin to the north.

Although this investigation qualitatively corroborated previous reports of *N. caninum* seroprevalence among cattle, water buffaloes and dogs in Thailand (Kashiwazaki *et al*., 2001; Chanlun *et al*., 2002; Chanlun *et al*., 2007; Jittapalapong *et al*., 2008; Arunvipas *et al*., 2012; Nam *et al*., 2012), quantitatively, the 9.1% overall individual host seroprevalence from the present study was less than half of the value based on previous reports and used to estimate sample size for the current study. However, two previous studies reported the seroprevalence of *N. caninum* among water buffaloes as 73.3% from a single farm in Chachoengsao province with cELISA (Wiengcharoen *et al*., 2010) and 4.5% in northeast Thailand with iscom ELISA (Nam *et al*., 2012). All of these investigations used different methods for detection of anti-*N. caninum* antibodies, which could partially explain the discrepant results. Additionally, the study reporting 73.3% seroprevalence was focused on a single farm, which corroborated the possibility of higher than average seroprevalence among water buffaloes on individual farms (Gennari *et al*., 2005; Campero *et al*., 2007; Meenakshi *et al*., 2007; Konrad *et al*., 2013;). However, the present report is focused on smallholder farms where water buffaloes serve as draft animals, thus requiring us to sample regions rather than individual farms with different management practices. Notably, results of the current study were similar to one of these previous reports, where an overall individual host seroprevalence of 4.5% was measured among

provinces that were all located in the Sakon Nakhon basin (Nam *et al*., 2012), because a seroprevalence of 4.2% was measured for the same basin in the current report. In Sakon Nakhon province, the only province sampled in both studies, the previous and current investigations detected 5.1% and 3.9% seroprevalence among individual water buffaloes, respectively, further illustrating the agreement between these reports where the study areas overlap. Thus, these results suggest that the higher seroprevalence in the Khorat basin is an important observation, underscoring the importance of risk factor analysis in identification of potential management issues, and warranting further investigations to further investigate differences among these basins in northeast Thailand.

There are at least two possible explanations for the higher seroprevalence values measured from the Khorat basin. First, exposure of water buffaloes to *N. caninum* could be associated with the presence of more rivers and their branches in the Khorat basin (Figure 17). These rivers are commonly used for agriculture, livestock husbandry, human and animal consumption and transportation. Therefore it is possible that *N. caninum* oocysts in these areas are less subject to desiccation due to moisture in the soil, that ungulates are more likely to be exposed to *N. caninum* oocysts when drinking and grazing in areas near a water source that is shared by canine definitive hosts of the parasite, and that transportation on these rivers can facilitate water buffalo exposure to feces from dogs indigenous to a broader geographic area. Second, *N. caninum* seroprevalence might be associated with animal migration from other countries that border the Thai Khorat basin to the south and to the east. Thus, both definitive and intermediate hosts could enter northeast Thailand from these neighboring countries that may not screen for or control livestock disease agents to the same degree. Importantly, only Thai provinces surround the provinces surveyed in the Sakon Nakhon Basin, while all of the provinces sampled in the Khorat Basin have at least one international border. Ubon Ratchathani, the province with the highest individual (16.7%) and second highest herd (30.5%) seroprevalence, has international borders with two other countries (Figure 23). There have also been reports of migration and illegal movement of animals into Thailand from neighboring

countries that might have less stringent public and animal health regulations (Senate, 2002; DFT, 2007).

The risk of host exposure to *N. caninum* appeared to increase with host age. This observation could be due to differences in immune systems among water buffalo age groups. However, some have reported seroprevalence rates that appeared less dependent of age (Gennari *et al*., 2005; Nasir *et al*., 2011), while others also measured the lowest seroprevalence among the youngest age group tested (Fujii *et al*., 2001; Konrad *et al*., 2013) and others reported trends that were similar to the current report (Guarino *et al*., 2000; Campero *et al*., 2007). Interestingly, one of the latter studies also reported higher seroprevalence at herd-level (82%) than among individual hosts (34.6%) (Guarino *et al*., 2000). These reports collectively suggest that seroprevalence differences among water buffalo age groups are not due to immune system differences alone, but that these differences can reflect the frequencies of horizontal and vertical transmission of *N. caninum* to water buffalo. For example, although vertical transmission occurs in buffaloes (Rodrigues *et al*., 2005) and is a frequent route of infection in cattle (Dubey *et al*., 2007), the increased risk of exposure with water buffalo age can be attributed to horizontal transmission of *N. caninum* from canine definitive to buffalo intermediate hosts in endemic regions. This conclusion is also supported by a previous report that the presence of dogs on farms might be an important risk factor bovine exposure to *N. caninum* in Thailand (Arunvipas *et al*., 2012).

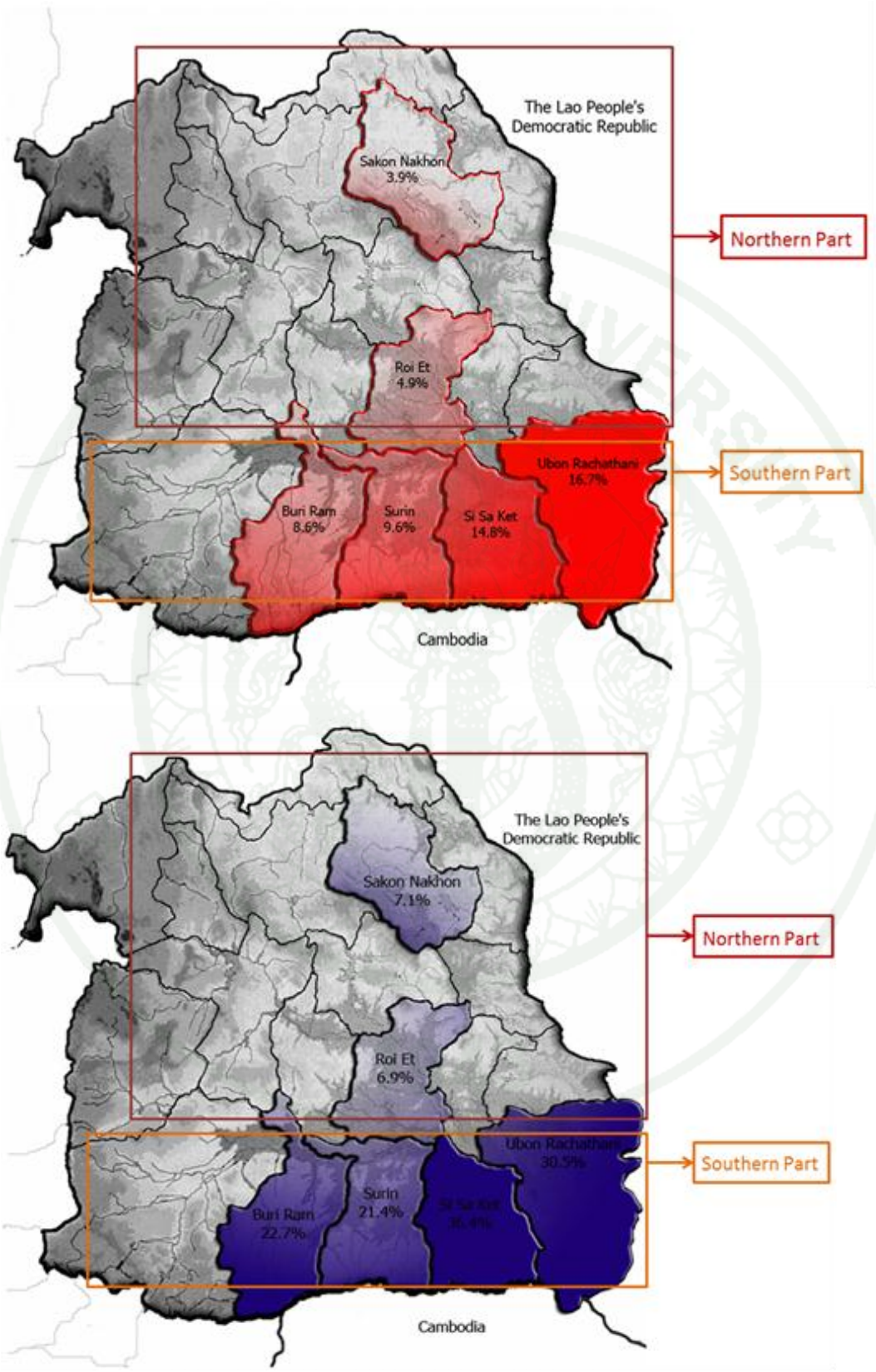


Figure 23 Individual and herd seroprevalence in study area

CONCLUSION

In conclusion, the seroprevalence of *N. caninum* was confirmed among water buffaloes in northeast Thailand. Location in the Khorat Basin and older host age were identified as putative risk factors, which, along with a greater herd than individual host prevalence, collectively suggest that horizontal transmission from canine definitive hosts is currently the most important means of exposure of water buffalo to *N. caninum* in northeast Thailand. Further work is warranted to evaluate the effects of river systems, international borders and the presence of infected dogs on exposure of water buffaloes to the parasite in this region. These results also underscore the importance of risk factor evaluations for effective control of neosporosis in different regions.

LITERATURE CITED

Abes, N.S. and B.P. Divina. 2008. Seroprevalence of *Neospora caninum* in Bulgarian Murrah Buffaloes and its Detection in Domestic Dogs from Buffalo Dairy Herds in Nueva Ecija, Philippines. **Philipp. J. Vet. Med.** 45: 30-38.

Ahn, H.J., S. Kim, D.Y. Kim and H.W. Nam. 2003. ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. **Korean J. Parasitol.** 41:175–177.

Alvarez-Garcia, G., E. Collantes-Fernandez, E. Costas, X. Rebordosa and L.M. Ortega-Mora. 2003. Influence of age and purpose for testing on the cut-off selection of serological methods in bovine neosporosis. **Vet. Res.** 34:341–352.

Anderson, M.L., P.C. Blanchard, B.C. Barr, J.P. Dubey, R.L. Hoffman and P.A. Conrad. 1991. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. **J. Am. Vet. Med. Assoc**. 198:241–244.

_____, A.G. Andrianarivo and P.A. Conrad. 2000. Neosporosis in cattle. **Anim. Reprod. Sci**. 60–61:417–431.

Antony, A. and N.B. Williamson. 2001. Recent advances in Understanding the epidemiology of *Neospora caninum* in cattle. **N. Z. Vet. J.** 49:42–47.

Arunvipas, P., T. Inpankaew and S. Jittapalapong. 2012. Risk factors of *Neospora caninum* infection in dogs and cats in dairy farms in Western Thailand. **Trop. Anim. Health Prod.** 44:1117-21.

Atkinson, R.A., R.W. Cook, L.A. Reddacliff, J. Rothwell, K.W. Broady, P.A.W. Harper and J.T. Ellis. 2000. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. **Aust. Vet. J.** 78:262–266.

Barber, J.S. 1998. Canine neosporosis. **Waltham Focus.** 8: 25 – 29.

_____and A.J. Trees. 1998. Naturally occurring vertical Transmission of *Neospora caninum* in dogs. **Int. J. Parasitol.** 28:57–64.

_____, C.E. Payne-Johnson and A.J. Trees. 1996. Distribution of *Neospora caninum* within the central nervous system and other tissues of six dogs with clinical neosporosis. **J. Small Anim. Pract**., inpress.

Barling, K.S., M. Sherman, M.J. Peterson, J.A. Thompson, J.W. McNeill, T.M. Craig and L.G. Adams. 2000. Spatial associations among density of cattle, abundance of wild canids, and seroprevalence to *Neospora caninum* in a population of beef calves. **J. Am. Vet. Med. Assoc**. 217:1361–1365.

_____, J.W. McNeill, J.C. Paschal, F.T. McCollum, T.M. Craig, L.G. Adams and J.A. Thompson. 2001. Ranch-management factors associated with antibody seropositivity for *Neospora caninum* in consignments of beef calves in Texas, USA. **Prev. Vet. Med.** 52: 53–61.

Barr, B.C., M.L. Anderson, J.P. Dubey and P.A. Conrad. 1991a. Neospora-like protozoal infections associated with bovine abortions. **Vet. Pathol**. 28:110-116.

_____, P.A. Conrad, J.P. Dubey and M.L. Anderson. 1991b. *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. **J. Vet. Diagn. Invest**. 3:39-46.

_____, _____, R. Breitmeyer, K. Sverlow, M.L. Anderson, J. Reynolds, A.E. Chauvet, J.P. Dubey and A.A. Ardans. 1993. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*- infected fetuses: four cases (1990-1992). **J. Am. Vet. Med. Assoc**. 202:113-117.

Bartels, C.J.M., W. Wouda and Y.H. Schukken. 1999. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). **Theriogenology**. 52:247–257.

_____, J.I. Arnaiz-Seco, A. Ruiz-Santa-Quitera, C. Bjorkman, J. Frossling, D. von Blumroder, F.J. Conraths, G. Schares, C. van Maanen, W. Wouda and L.M. Ortega-Mora. 2006. Supranational comparison of *Neospora caninum* seroprevalences in cattle in Germany, the Netherlands, Spain and Sweden. **Vet. Parasitol**. 137:17–27.

Basso, W., L. Venturini, M.C. Venturini, P. Moore, M. Rambeau, J.M. Unzaga, C. Campero, D. Bacigalupe and J.P. Dubey. 2001a. Prevalence of *Neospora caninum* infection in dogs from beef-cattle farms, dairy farms, and from urban areas of Argentina. **J. Parasitol**. 87:906–907.

_____, _____, _____, D.E. Hill, O.C.H. Kwok, S.K. Shen and J.P. Dubey. 2001b. First isolation of *Neospora caninum* from the feces of a naturally infected dog. **J. Parasitol**. 87:612–618.

Baszler, T.V., L.J.C. Gay, M.T. Long and B.A. Mathison. 1999. Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. **J. Clin. Microbiol**. 37:4059–4064.

Bergeron, N., G. Fecteau, A. Villeneuve, C. Girard and J. Pare. 2001. Failure of dogs to shed oocysts after being fed bovine fetuses naturally infected by *Neospora caninum*. **Vet. Parasitol.** 97:145–152.

Bjerkas, I., S. F. Mohn and J. Presthus. 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. **Z. Parasitenkd**. 70:271–274.

Bjorkman, C. and A. Uggla. 1999. Serological diagnosis of *Neospora caninum* infection. **Int. J. Parasitol**. 29:1497–1507.

Bjorkman, C., A. Lunden, J. Holmdahl, J. Barber, A.J. Trees, and A. Uggla. 1994. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. **Parasite Immunol.** 16:643–648.

Bryan, L.A., A. A. Gajadhar, J. P. Dubey and D.M. Haines, 1994. Bovine neonatal encephalomyelitis associated with a *Neospora sp.* protozoan. **Can. Vet. J.** 35: 111-113.

Buxton, D., S. Wright, S.W. Maley, A.G. Rae, A. Lunden and E.A. Innes. 2001. Immunity to experimental neosporosis in pregnant sheep. **Parasite Immunol**. 23:85–91.

Caetano-da-Silva, A., I. Ferre, E. Collantes-Fernandez, V. Navarro, G. Aduriz, C. Ugarte-Garagalza and L.M. Ortega-Mora. 2004. Occasional detection of *Neospora caninum* DNA in frozen extended semen from naturally infected bulls. **Theriogenology**. 62:1329–1336.

Campero, C.M. and A. Perez. 2007. Occurrence of antibodies against *Neospora caninum* in water buffaloes (*Bubalus bubalis*) on four ranches in Corrientes province, Argentina. **Vet. Parasitol**. 150:155-158.

Canada, N., C.S. Meireles, P. Ferreira, J.M.C. da Costa and A. Rocha. 2006. Artificial insemination of cows with semen in vitro contaminated with *Neospora caninum* tachyzoites failed to induce neosporosis. **Vet. Parasitol.** 139:109-114.

_____, _____, A. Rocha, J.M. Correia da Costa, M.W. Erickson and J.P. Dubey. 2002. Isolation of viable *Toxoplasma gondii* from naturally-infected aborted bovine fetuses. **J. Parasitol**. 88:1247–1248.

Chantalakhana, C. 2001. Urgent need in buffalo development for food security and self-sufficiency. In Proceedings of the National Workshop on Swamp Buffalo Development. Hanoi, Vietnam (December, 17–18 2001)

Chanlun, A., U. Emanuelson, J. Frossling, S. Aiumlamai and C. Bjorkman. 2007. A longitudinal study of seroprevalence and seroconversion of *Neospora caninum* infection in dairy cattle in northeast Thailand. **Vet. Parasitol.** 146: 242-248.

_____, K. Naslund, S. Aiumlamai and C. Bjorkman. 2002. Use of bulk milk for detection of *Neospora caninum* infection in dairy herds in Thailand. **Vet. Parasitol**. 110:35–44.

Chi, J., J.A. VanLeeuwen, A. Weersink and G.P. Keefe. 2002. Direct production losses and treatment costs from bovine viral diarrhea virus, bovine leukosis virus, *Mycobacterium avium* subspecies paratuberculosis, and *Neospora caninum*. **Prev. Vet. Med**. 55:137–153.

Conrad, P.A., K. Sverlow, M. Anderson, J. Rowe, R. BonDurant, G. Tuter, R. Breitmeyer, C. Palmer, M. Thurmond, A. Ardans, J.P. Dubey, G. Duhamel and B. Barr. 1993. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. **J. Vet. Diagn. Invest**. 5:572–578.

Corbellini, L.G., D. Driemeier, C.F.E. Cruz, L.F.P. Gondim and V. Wald. 2002. Neosporosis as a cause of abortion in dairy cattle in Rio Grande do Sul, southern Brazil. **Vet. Parasitol**. 103:195–202.

_____, D.R. Smith, C.A. Pescador, M. Schmitz, A. Correa, D.J. Steffen and D. Driemeier. 2006. Herd-level risk factors for *Neospora caninum* seroprevalence in dairy farms in southern Brazil. **Prev. Vet. Med.** 74:130–141.

Czerniawska-Piatkowska, E., E. Chocilowicz and M. Szewczuk. 2010. Biology of *Bubalus bubalis*. **Ann. Anim. Sci.** 10:107–115.

Davison, H.C., N.P. French and A.J. Trees. 1999. Herd-specific and age-specific seroprevalence of *Neospora caninum* in 14 British dairy herds. **Vet. Rec**. 144:547–550.

_____, C.S. Guy, J W. McGarry, F. Guy, D.J.L. Williams, D.F. Kelly and A.J. Trees. 2001. Experimental studies on the transmission of *Neospora caninum* between cattle. **Res. Vet. Sci.** 70:163–168.

de Souza, S.L.P., J.S. Guimaraes, F. Ferreira, J.P. Dubey and S.M. Gennari. 2002. Prevalence of *Neospora caninum* antibodies in dogs from dairy cattle farms in Parana, Brazil. **J. Parasitol**. 88:408–409.

Department of Foreign Trade, Ministry of Commerce. 2007. **Beef products. (in Thai)** Available Source: 203.113.25.97/the_files/$$16/level3/Bovine.doc, October 1, 2012

Department of Livestock Development. 2010. **Cattle and Buffalo population. (in Thai)** Available Source: http://www.dld.go.th, October 1, 2012

Dijkstra, T., H. W. Barkema, M. Eysker, J. W. Hesselink and W. Wouda. 2002. Natural transmission routes of *Neospora caninum* between farm dogs and cattle. **Vet. Parasitol**. 105:99–104.

_____, _____, _____ and W. Wouda. 2001. Evidence Of post-natal transmission of *Neospora caninum* in Dutch dairy herds. **Int. J. Parasitol**. 31:209–215.

Dubey, J.P. 1992. A review of *Neospora caninum* and Neospora like infections in animals. **J. Protozool Res**. 2:40-52.

Dubey, J.P. 1993. *Toxoplasma, Neospora, Sarcocystis*, and other tissue cyst-forming coccidia of humans and animals, pp. 1-158. *In* J. P. Kreier ed. **Parasitic Protozoa**. Academic Press, London.

_____ 1999a. Neosporosis in cattle: biology and economic impact. **J. Am. Vet. Med. Assoc.** 214:1160–1163.

_____ 1999b. Recent advances in *Neospora* and neosporosis. **Vet. Parasitol**. 84: 349–367.

_____ 2003. Neosporosis in cattle. **J. Parasitol**. 89:S42–S46.

_____ and A. de Lahunta. 1993. Neosporosis associated congenital limb deformities in a calf. **Appl. Parasitol**. 34:229-233.

_____ and D.S. Lindsay. 1993. Neosporosis. **Parasitol. Today**. 9:452-458.

_____ and _____ 1996. A review of *Neospora caninum* and neosporosis. **Vet. Parasitol**. 67:1–59.

_____ and _____ 2000. Gerbils (Meriones unguiculatus) are highly susceptible to oral infection with *Neospora caninum* oocysts. **Parasitol. Res.** 86:165-168.

_____ and G. Schares. 2006. Diagnosis of bovine neosporosis. **Vet. Parasitol**. 140:1–34

_____, B. Abbitt, M. J. Topper and J. F. Edwards. 1998a. Hydrocephalus associated with *Neospora caninum* infection in an aborted bovine fetus. **J. Comp. Pathol**. 118:169-173.

Dubey, J.P., B.C. Barr, J.R. Barta, I. Bjerkas, C. Bjorkman, B.L. Blagburn, D.D. Bowman, D. Buxton, J.T. Ellis, B. Gottstein, A. Hemphill, D.E. Hill, D.K. Howe, M.C. Jenkins, Y. Kobayashi, B. Koudela, A.E. Marsh, J.G. Mattsson, M.M. McAllister, D. Modry, Y. Omata, L.D. Sibley, C.A. Speer, A.J. Trees, A. Uggla, S.J. Upton, D.J.L. Williams and D.S. Lindsay. 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. **Int. J. Parasitol**. 32:929–946.

_____, D. Buxton and W. Wouda. 2006. Pathogenesis of bovine neosporosis. **J. Comp. Pathol**. 134:267–289.

_____, J.L. Carpenter, C.A. Speer, M.J. Topper and A. Uggla. 1988b. Newly recognized fatal protozoan disease of dogs. **J. Am. Vet. Med. Assoc.** 192:1269–1285.

_____, A.L. Hattel, D.S. Lindsay and M.J. Topper. 1988a. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. **J. Am. Vet. Med. Assoc.** 193:1259–1263.

_____, H.P.A. Hughes, H.S. Lillehoj, H.R. Gamble and B.L. Munday. 1987. Placental transfer of specific antibodies during ovine congenital toxoplasmosis. **Am. J. Vet. Res.** 48:474-476.

_____, E.B. Janovitz and A.J. Skowronek. 1992b. Clinical neosporosis in a 4-week-old Hereford calf. **Vet. Parasitol**. 43:137-141.

_____, A. Koestner and R.C. Piper. 1990b. Repeated Transplacental transmission of *Neospora caninum* in dogs. **J. Am. Vet. Med. Assoc**. 197: 857–860.

_____, C.W. Leathers and D.S. Lindsay. 1989. *Neospora caninum*-like protozoon associated with fatal myelitis in newborn calves. **J. Parasitol**. 75:146-148.

Dubey, J.P., D.S. Lindsay and M.R. Lappin. 2009. Toxoplasmosis and Other Intestinal Coccidial Infections in Cats and Dogs. **Vet. Clin. Small Anim.** 39: 1009–1034.

_____, F.L.J. Metzger, A.L. Hattel, D.S. Lindsay and D.L. Fritz. 1995. Canine cutaneous neosporosis: clinical improvement with clindamycin. **Vet. Dermatol**. 6:37-43.

_____, S. Romand, M. Hilali, O.C.H. Kwok and P. Thulliez. 1998b. Seroprevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in water buffaloes (*Bubalus bubalis*) from Egypt. **Int. J. Parasitol**. 28:527–529.

_____, G. Schares and L.M. Ortega-Mora. 2007. Epidemiology and Control of Neosporosis and *Neospora caninum*. **Clin. Microbiol. Rev.** 20: 323-367.

_____, C. Sreekumar, E. Knickman, K.B. Miska, M.C.B. Vianna, O.C.H. Kwok, D.E. Hill, M.C. Jenkins, D.S. Lindsay and C.E. Greene. 2004. Biologic, morphologic, and molecular characterisation of *Neospora caninum* isolates from littermate dogs. **Int. J. Parasitol**. 34:1157-1167.

Dyer, R.M., M.C. Jenkins, O.C.H. Kwok, L.W. Douglas and J.P. Dubey. 2000. Serologic survey of *Neospora caninum* infection in a closed dairy cattle herd in Maryland: risk of serologic reactivity by production groups. **Vet. Parasitol**. 90:171–181.

Faarungsang, S. 2003. Thai swamp buffalo general information. pp. 3-17. *In* H.L. Chang and Y.C. Huang, eds. **The Relationship Between Indigenous Animals and Humans in APEC Region.** The Chinese Society of Animal Science press, Taiwan.

Ferre, I., G. Aduriz, I. del-Pozo, J. Regidor-Cerrillo, R. Atxaerandio, E. Collantes-Fernandez, A. Hurtado, C. Ugarte-Garagalza and L.M. Ortega-Mora. 2005. Detection of *Neospora caninum* in the semen and blood of naturally infected bulls. **Theriogenology.** 63:1504–1518.

Fritz, D., C. George, J.P. Dubey, A.J. Trees, J.S. Barber, C.L. Hopfner, S. Mehaut, J.L. Le Net and L. Longeart. 1997. *Neospora caninum*: associated nodular dermatitis in a middle-aged dog. **Canine Pract.** 22:21–24.

Frossling, J., A. Uggla and C. Bjorkman. 2005. Prevalence and transmission of *Neospora caninum* within infected Swedish dairy herds. **Vet. Parasitol.** 128:209-218.

Fujii, T. U., N. Kasai, S. M. Nishi, J. P. Dubey and S. M. Gennari. 2001. Seroprevalence of *Neospora caninum* in female water buffaloes (*Bubalus bubalis*) from the southeastern region of Brazil. **Vet. Parasitol**. 99:331–334.

Gennari, S. M., A. A. R. Rodgrigues, R. B. Viana and E. C. Cardoso. 2005. Occurrence of anti-*Neospora caninum* antibodies in water buffaloes (*Bubalus bubalis*) from the northern region of Brazil. **Vet. Parasitol.** 134:169–171.

Gondim, L.F.P., M.M. McAllister, R.C. Anderson-Sprecher, C. Bjorkman, T.F. Lock, L.D. Firkins, L. Gao and W.R. Fischer. 2004a. Transplacental transmission and abortion in cows administered *Neospora caninum* oocysts. **J. Parasitol.** 90:1394-1400.

_____, _____ and L. Gao. 2005. Effects of host maturity and prior exposure history on the production of *Neospora caninum* oocysts by dogs. **Vet. Parasitol**. 134:33–39.

_____, _____, W. C. Pitt and D. E. Zemlicka. 2004b. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. **Int. J. Parasitol**. 34:159–161.

Gondim, L.F.P., I. F. Sartor, M. Hasegawa and I. Yamane. 1999. Seroprevalence of *Neospora caninum* in dairy cattle in Bahia, Brazil. **Vet. Parasitol**. 86:71–75.

Gonzalles, L., D. Buxton, R. Atxaerandio, G. Aduriz, S. Maley, J. C. Marco and L.A. Cuervo. 1999. Bovine abortion associated with *Neospora caninum* in northern Spain. **Vet. Rec**. 144:145-150.

Gottstein, B., B. Hentrich, R. Wyss, B. Thur, A. Busato, K.D.C. Stark and N. Muller. 1998. Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. **Int. J. Parasitol**. 28:679–691.

Guarino, A., G. Fusco, G. Savini, G. Di Francesco and G. Cringoli. 2000. Neosporosis in water buffalo (Bubalus bubalis) in southern Italy. **Vet. Parasitol**. 91:15–21.

Guy, C.S., D.J.L. Williams, D.F. Kelly, J.W. McGarry, F. Guy, C. Bjorkman, R.F. Smith and A.J. Trees. 2001. *Neospora caninum* in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody. **Vet. Rec**. 149:443–449.

Haddad, J.P.A., I.R. Dohoo and J.A. VanLeewen. 2005. A review of *Neospora caninum* in dairy and beef cattle—a Canadian perspective. **Can. Vet. J.** 46:230–243.

Hajikolaei M.R.H., S. Goraninejad, H. Hamidinejat, M. Ghorbanpour and R. Paryab. 2007. Occurrence of *Neospora caninum* antibodies in water buffaloes (Bubalus bubalis) from the South-Western region of Iran. **Bull Vet. Inst. Pulawy.** 51: 233-235.

Hasler, B., G. Regula, K.D.C. Stark, H. Sager, B. Gottstein and M. Reist. 2006. Financial analysis of various strategies for the control of *Neospora caninum* in dairy cattle in Switzerland. **Prev. Vet. Med**. 77:230–253.

Hemphill A. and B. Gottstein. 2000. A European perspective on *Neospora caninum*. **Int. J. Parasitol**. 30:877–924.

Hernandez, J., C. Risco and A. Donovan. 2001. Association between exposure to *Neospora canin*um and milk production in dairy cows. **J. Am. Vet. Med. Assoc**. 219:632–635.

Hill, D.E., S. Liddell, M.C. Jenkins and J.P. Dubey. 2001. Specific detection of *Neospora caninum* oocyst in fecal samples from experimentally-infected dogs using the polymerase chain reaction. **J. Parasitol.** 87:395–398.

Ho, M.S.Y., B.C. Barr, J.D. Rowe, M.L. Anderson, K.W. Sverlow, A. Packham, A.E. Marsh and P.A. Conrad. 1997. Detection of *Neospora sp.* from infected bovine tissues by PCR and probe hybridization. **J. Parasitol**. 83:508–514.

Hobson, J.C., T.F. Duffield, D. Kelton, K. Lissemore, S.K. Hietala, K.E. Leslie, B. McEwen, G. Cramer and A.S. Peregrine. 2002. *Neospora caninum* serostatus and milk production of Holstein cattle. **J. Am. Vet. Med. Assoc**. 221:1160–1164.

_____, _____, _____, _____, _____, _____, _____ and A. S. Peregrine. 2005. Risk factors associated with *Neospora caninum* abortion in Ontario Holstein dairy herds. **Vet. Parasitol.** 127:177–188.

Huong, L.T.T., B.L. Ljungstrom, A. Uggla and C. Bjorkman. 1998. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes in southern Vietnam. **Vet. Parasitol**. 75:53–57.

International Buffalo Information Centre, 2009. **About Water Buffalo. (in Thai)** Available Source: http://ibic.lib.ku.ac.th/, October 30, 2012.

Jittapalapong, S., A. Sangwaranond, T. Inpankaew, C. Phasuk, N. Pinyopanuwat, W. Chimnoi, C. Kengradomkij, S. Saebgow, P. Pumhom, P. Arunwipat, T. Anakewit and I. D. Robertson. 2008. Seroprevalence of *Neospora caninum* infections of dairy cows in the north-east of Thailand. **Kasetsart J**. 42:61-66.

Jensen, A.M., C. Bjorkman, A.M. Kjeldsen, A. Wedderkopp, C. Willadsen, A. Uggla and P. Lind. 1999. Associations of *Neospora caninum* seropositivity with gestation number and pregnancy outcome in Danish dairy herds. **Prev. Vet. Med**. 40:151–163.

Kashiwazaki, Y., S. Pholpark, A. Charoenchai, C. Polsar, S. Teeverapanya and M. Pholpark. 2001. Postnatal neosporosis in dairy cattle in northeast Thailand. **Vet. Parasitol.** 94:217–220

Kim, J.H., M.S. Kang, B.C. Lee, W.S. Hwang, C.W. Lee, B.J. So, J.P. Dubey and D.Y. Kim. 2003. Seroprevalence of antibodies to *Neospora caninum* in dogs and raccoon dogs in Korea. **Korean J. Parasitol**. 41:243– 245.

King, J.S., B. McAllan, D.S. Spielman, S.A. Lindsay, L. Hurkova-Hofmannova, A. Hartigan, S.E. Al-Qassab, J.T. Ellis and J. Slapeta. 2011. Extensive production of *Neospora caninum* tissue cysts in a carnivorous marsupial succumbing to experimental neosporosis. **Vet. Res.** 42:75.

Koiwai, M., T. Hamaoka, M. Haritani, S. Shimizu, T. Tsutsui, M. Eto and I. Yamane. 2005. Seroprevalence of *Neospora caninum* in dairy and beef cattle with reproductive disorders in Japan. **Vet. Parasitol**. 130:15–18.

Konnai, S., C.N. Mingala, M. Sato, N.S. Abes, F.A. Venturina, C.A. Gutierrez, T. Sano, Y. Omata, L.C. Cruz, M. Onuma and K. Ohashi. 2008. A survey of abortifacient infectious agents in livestock in Luzon, the Philippines, with emphasis on the situation in a cattle herd with abortion problems. **Acta Trop.** 105: 269-273.

Konrad, J.L., L.M. Campero, G.S. Caspe, B. Brihuega, G. Draghi, D.P. Moore, G.A. Crudeli, M.C. Venturini and C.M. Campero. 2013. Detection of antibodies against *Brucella abortus*, *Leptospira spp*., and Apicomplexa protozoa in water buffaloes in the Northeast of Argentina. **Trop. Anim. Health Prod.** 45: 1751–1756.

Kumar, S., M. Nagarajan, J.S. Sandhu and V. Behl. 2007. Phylogeography and domestication of Indian river buffalo. **BMC Evol. Biol.** 7:186.

Kyaw, T., P. Virakul, M. Muangyai and W. Banlunara. 2003. First identification of *Neospora caninum* in Thailand. **Thai. J. Vet. Med.** 33:97–102.

_____, _____, _____ and J. Suwimonteerabutr. 2004. *Neospora caninum* seroprevalence in dairy cattle in central Thailand. **Vet. Parasitol**. 121:255–263.

La Perle K.M., F. Del Piero, R.F. Carr, C. Harris and P.C. Stromberg. 2001. Cutaneous neosporosis in two adult dogs on chronic immunosuppressive therapy. **J. Vet. Diagn. Invest.** 13:252-5.

Lindsay, D.S. and J.P. Dubey. 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. **Am. J. Vet. Res**. 50:1981-1983.

_____ and _____. 1990. Infections in mice with tachyzoites and bradyzoites of *Neospora caninum* (Protozoa: Apicomplexa). **J. Parasitol.** 76:410-413.

_____, B.L. Blagburn and J.P. Dubey. 1992. Factors affecting the survival of *Neospora caninum* bradyzoites in murine tissues. **J. Parasitol.** 78:70-72.

_____, J.P. Dubey and R.B. Duncan. 1999. Confirmation that the dog is a definitive host for *Neospora caninum*. **Vet. Parasitol**. 82:327–333.

Lindsay, D.S., C.A. Speer, M.A. Toivio-Kinnucan, J.P. Dubey and B.L. Blagburn. 1993. Use of infected cultured cells to compare ultrastrnctural features of *Neospora caninum* from dogs and *Toxoplasma gondii*. **Am. J. Vet. Res**. 54:103-106.

Locatelli-Dittrich, R., V.T. Soccol, R.R.T.B. Richartz, M.E. Gasino- Joineau, R. Vinne and R.D. Pinckney. 2001. Serological diagnosis of neosporosis in a herd of dairy cattle in southern Brazil. **J. Parasitol**. 87:1493–1494.

Lopez-Gatius, F., M. Lopez-Bejar, K. Murugavel, M. Pabon, D. Ferrer and S. Almeria. 2004. *Neospora*-associated abortion episode over a 1-year period in a dairy herd in north-east Spain. **J. Vet. Med.** 51:348-352.

Macaldowie, C., S.W. Maley, S. Wright, P. Bartley, I. Esteban- Redondo, D. Buxton and E. Innes. 2004. Placental pathology associated with fetal death in cattle inoculated with *Neospora caninum* by two different routes in early pregnancy. **J. Comp. Pathol**. 131:142-156.

Mainar-Jaime, R.C., M.C. Thurmond, B. Berzal-Herranz and S.K. Hietala. 1999. Seroprevalence of *Neospora caninum* and abortion in dairy cows in northern Spain. **Vet. Rec**. 145:72–75.

Mark, T. T. 2009. Infectious Neuromuscular Diseases of Dogs and Cats. **Top. Companion. Anim. Med.** 24:209–220.

McAllister, M.M., E.M. Huffman, S.K. Hietala, P.A. Conrad, M.L. Anderson and M.D. Salman. 1996. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. **J. Vet. Diagn. Investig**. 8:355-357.

McAllister, M.M., J.P. Dubey, D.S. Lindsay, W.R. Jolley, R.A. Wills and A.M. McGuire. 1998. Dogs are definitive hosts of *Neospora caninum*. **Int. J. Parasitol**. 28:1473–1478.

McElwain, T., D.P. Knowles and C.W. Leathers. 1987. Myelitis associated with protozoal infection in newborn calves. **J. Am. Vet. Med. Assoc.** 191:1599-1600.

McGarry, J.W., C.M. Stockton, D.J.L. Williams and A.J. Trees. 2003. Protracted shedding of oocysts of *Neospora caninum* by a naturally infected foxhound. **J. Parasitol**. 89:628–630.

McInnes, L.M., P. Irwin, D.G. Palmer and U.M. Ryan. 2006. In vitro isolation and characterization of the first canine *Neospora caninum* isolate in Australia. **Vet. Parasitol.** 137:355–363.

Medina, L., C. Cruz-Vazquez, T. Quezada, E. Morales and Z. Garcia- Vazquez. 2006. Survey of *Neospora caninum* infection by nested PCR in aborted fetuses from dairy farms in Aguascalientes, Mexico. **Vet. Parasitol**. 136:187–191.

Meenakshi, K.S. Sandhu, M.S. Ball, H. Kumar, S. Sharma, P.K. Sidhu, C. Sreekumar and J.P. Dubey. 2007. Seroprevalence of *Neospora caninum* antibodies in cattle and water buffaloes in India. **J. Parasitol**. 93:1374–1377.

Moore, D.P., C.M. Campero, A.C. Odeon, M.A. Posso, D. Cano, M.R. Leunda, W. Basso, M.C. Venturini and E. Spath. 2002. Seroepidemiology of beef and dairy herds and fetal study of *Neospora caninum* in Argentina. **Vet. Parasitol**. 107:303–316.

Morales, E., F. J. Trigo, F. Ibarra, E. Puente and M. Santacruz. 2001a. Seroprevalence study of bovine neosporosis in Mexico. **J. Vet. Diagn. Investig**. 13:413–415.

Morales, E., F. J. Trigo, F. Ibarra, E. Puente and M. Santacruz.2001b. Neosporosis in Mexican dairy herds: lesions and immunohistochemical detection of *Neospora caninum* in fetuses. **J. Comp. Pathol.** 125:58–63.

Moskwa, B., W. Cabaj, K. Pastusiak and J. Bien. 2003. The suitability of milk in detection of *Neospora caninum* infection in cows. **Acta Parasitol**. 48:138–141.

_____, K. Pastusiak, J. Bien and W. Cabaj. 2007. The first detection of *Neospora caninum* DNA in the colostrum of infected cows. **Parasitol. Res.** 100:633-636.

Nanda, A.S. and T. Nakao. 2003. Role of buffalo in the socioeconomic development of rural Asia: Current status and future prospectus. **J. Anim. Sci.** 74:443-455.

Nam, N.H., C. Aran, K. Kwankate and A. Suneerat. 2012. Seroprevalence of *Neospora caninum* in swamp buffaloes and beef cattle in the Northeast of Thailand. **Thai J. Vet. Med.** 42: 213-218.

Nasir, A., M. Ashraf, M.S. Khan, T. Yaqub, A. Javeed, M. Avais and F. Akhtar. 2011. Seroprevalence of *Neospora caninum* in dairy buffaloes in Lahore District, Pakistan. **J Parasitol.** 97:541-543.

Nietfeld, J.C., J.P. Dubey, M.L. Anderson, M.C. Libal, M.J. Yaeger and R.D. Neiger. 1992. *Neospora*-like protozoan infection as a cause of abortion in dairy cattle. **J. Vet. Diagn. Invest**. 4: 223-226.

O'Toole, D. and M. Jeffrey, 1987. Congenital sporozoan encephalomyelitis in a calf. **Vet. Rec.** 121: 563-566.

Okeoma, C.M., N.B. Williamson, W.E. Pomroy, K.M. Stowell and L. M. Gillespie, 2004a. The use of PCR to detect *Neospora caninum* DNA in the blood of naturally infected cows. **Vet. Parasitol**. 122:307–315

_____, _____, _____, _____ and _____. 2004b. Isolation and molecular characterization of *Neospora caninum* in cattle in New Zealand. **N. Z. Vet. J.** 52:364–370.

Ooi, H.K., C.C. Huang, C.H. Yang and S.H. Lee. 2000. Serological survey and first finding of *Neospora caninum* in Taiwan, and the detection of its antibodies in various body fluids of cattle. **Vet. Parasitol**. 90:47–55.

Ortega, Y.R., M.P. Torres and K.D. Mena. 2007. Presence of *Neospora caninum* specific antibodies in three dairy farms in Georgia and two in Texas. **Vet. Parasitol**. 144:353–355.

Ortega-Mora, L. M., A. Fernandez-Garcia and M. Gomez-Bautista. 2006. Diagnosis of bovine neosporosis: Recent advances and perspectives. **Acta Parasitol**. 51: 1-14.

_____, I. Ferre, I. del Pozo, A. Caetano da Silva, E. Collantes-Fernandez, J. Regidor-Cerrillo, C. Ugarte-Garagalza and G. Aduriz. 2003. Detection of *Neospora caninum* in semen of bulls. **Vet. Parasitol**. 117:301–308.

Ortuno, A., J. Castella and S. Almeria. 2002. Seroprevalence of antibodies to *Neospora caninum* in dogs from Spain. **J. Parasitol**. 88:1263–1266.

Osawa, T., J. Wastling, L. Acosta, C. Ortellado, J. Ibarra and E. A. Innes. 2002. Seroprevalence of *Neospora caninum* infection in dairy and beef cattle in Paraguay. **Vet. Parasitol**. 110:17–23.

Otranto, D., A. Llazari, G. Testini, D. Traversa, A.F. di Regalbono, M. Badan and G. Capelli. 2003. Seroprevalence and associated risk factors of neosporosis in beef and dairy cattle in Italy. **Vet. Parasitol**. 118:7–18.

Otter, A., M. Jeffrey, S.F.E. Scholes, B. Helmick, J.W. Wilesmith and A.J. Trees. 1997. Comparison of histology with maternal and fetal serology for the diagnosis of abortion due to bovine neosporosis. **Vet. Rec**. 141:487–489.

Ould-Amrouche, A., F. Klein, C. Osdoit, H.O. Mohamed, A. Touratier, M. Sanaa and J.P. Mialot. 1999. Estimation of *Neospora caninum* seroprevalence in dairy cattle from Normandy, France. **Vet. Res.** 30:531–538.

Padungtod, P. 2007. **Veterinary Epidemiology.** Morchoawban Publishers, Bangkok.

Paradies, P., G. Capelli, G. Testini, C. Cantacessi, A.J. Trees and D. Otranto. 2007. Risk factors for canine neosporosis in farm and kennel dogs in southern Italy. **Vet. Parasitol**. 145:240-244.

Pare, J., G. Fecteau, M. Fortin and G. Marsolais. 1998. Seroepidemiologic study of *Neospora caninum* in dairy herds. J. Am. **Vet. Med. Assoc.** 213:1595–1598.

_____, S.K. Hietala and M.C. Thurmond. 1995. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora sp*. infection in cattle. **J. Vet. Diagn. Investig**. 7:273–275.

_____, M.C. Thurmond, and S.K. Hietala. 1994. Congenital Neospora infection in dairy cattle. **Vet. Rec.** 134:531–532.

_____, _____ and _____. 1996. Congenital Neospora caninum infection in dairy cattle and associated calfhood mortality. **Can. J. Vet. Res.** 60:133-139.

Parish, S.M., L. Maag-Miller, T.E. Besser, J.P. Weidner, T. McEIwain, D.P. Knowles and C.W. Leathers. 1987. Myelitis associated with protozoal infection in newborn calves. **J. Am. Vet. Med. Assoc**. 191:1599-1600.

Patitucci, A.N., M.R. Alley, B.R. Jones and W.A.G. Charleston. 1997. Protozoal encephalomyelitis of dogs involving *Neospora caninum* and *Toxoplasma gondii* in New Zealand. **N. Z. Vet. J**. 45:231–235.

Pereira-Bueno, J., A. Quintanilla-Gozalo, V. Perez-Perez, A. Espi- Felgueroso, G. Alvarez, E. Collantes-Fernandez and L.M. Ortega- Mora. 2003. Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. **Vet. Parasitol**. 111:143–152.

Perl, S., S. Harrus, C. Satuchne (Goldvaser), B. Yakobson and D. Haines. 1998. Cutaneous neosporosis in a dog in Israel. **Vet. Parasitol**. 79:257–261.

Peters, M., E. Lutkefels, A.R. Heckeroth and G. Schares. 2001a. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. **Int. J. Parasitol**. 31:1144–1148.

_____, F. Wagner and G. Schares. 2000. Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. **Parasitol. Res**. 86:1-7.

Poli, A., F. Mancianti, M. A. Carli, M. C. Stroscio and L. Kramer. 1998. *Neospora caninum* infection in a Bernese cattle dog from Italy. **Vet. Parasitol**. 78:79–85.

Reichel, M.P. 1998. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. **N. Z. Vet. J.** 46:38.

Reichel, M.P. 2000. *Neospora caninum* infections in Australia and New Zealand. **Aust. Vet. J.** 78:258–261.

_____ and D. U. Pfeiffer. 2002. An analysis of the performance characterisics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. **Vet. Parasitol**. 107:197–207.

Rinaldi, L., G. Fusco, V. Musella, V. Veneziano, A. Guarino, R. Taddei and G. Cringoli. 2005. *Neospora caninum* in pastured cattle: determination of climatic, environmental, farm management and individual animal risk factors using remote sensing and geographical information systems. **Vet. Parasitol**. 128:219–230.

Rodrigues, A.A.R., S.M. Gennari, D.M. Aguiar, C. Sreekumar, D.E. Hill, K.B. Miska, M.C.B. Vianna and J.P. Dubey. 2004. Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. **Vet. Parasitol**. 124:139–150.

_____, _____, V.S.O. Paula,D.M. Aguiar, T.U. Fujii, W. Starke-Buzetti, R.Z. Machado and J.P. Dubey. 2005. Serological response to *Neospora caninum* in experimentally and naturally infected water buffaloes (*Bubalis bubalis*). **Vet. Parasitol.** 129:21–24.

Romero, J.J., E. Perez, G. Dolz and K. Frankena. 2002. Factors associated with *Neospora caninum* serostatus in cattle of 20 specialized Costa Rican dairy herds. **Prev. Vet. Med**. 53:263–273.

_____, S. Van Breda, B. Vargas, G. Dolz and K. Frankena. 2005. Effect of neosporosis on productive and reproductive performance of dairy cattle in Costa Rica. **Theriogenology**. 64:1928–1939.

Sager, H., I. Fischer, K. Furrer, M. Strasser, A. Waldvogel, P. Boerlin, L. Audige and B. Gottstein. 2001. A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. **Vet. Parasitol**. 102:1–15.

Sanchez, G.F., E. Morales, M.J. Martinez and J.F. Trigo. 2003. Determination and correlation of anti-*Neospora caninum* antibodies in dogs and cattle from Mexico. **Can. J. Vet. Res**. 67:142–145.

Sanderson, M.W., J.M. Gay and T.V. Baszler. 2000. *Neospora Caninum* seroprevalence and associated risk factors in beef cattle in the northwestern United States. **Vet. Parasitol**. 90:15–24.

Sawada, M., C.H. Park, H. Kondo, T. Morita, A. Shimada, I. Yamane and T. Umemura. 1998. Serological survey of antibody to *Neospora caninum* in Japanese dogs. **J. Vet. Med. Sci.** 60:853–854.

_____, H. Kondo, Y. Tomioka, C. H. Park, T. Morita, A. Shimada and T. Umemura. 2000. Isolation of *Neospora caninum* from the brain of a naturally infected adult dairy cow. **Vet. Parasitol**. 90:247–252.

Schares, G., A. Barwald, C. Staubach, R. Wurm, M. Rauser, F. J. Conraths and C. Schroeder. 2004. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. **Vet. Parasitol**. 120:55–63.

_____, _____, _____, M. Ziller, D. Kloss, R. Wurm, M. Rauser, R. Labohm, K. Drager, W. Fasen, R.G. Hess and F.J. Conraths. 2003. Regional distribution of bovine *Neospora caninum* infection in the German state of Rhineland-Palatinate modeled by logistic regression. **Int. J. Parasitol**. 33:1631–1640.

Schares, G., A.O. Heydorn, A. Cuppers, F.J. Conraths and H. Mehlhorn. 2001. *Hammondia heydorni*-like oocysts shed by a naturally infected dog and *Neospora caninum* NC-1 cannot be distinguished. **Parasitol. Res.** 87:808-816.

_____, N. Pantchev, D. Barutzki, A.O. Heyddorn, C. Bauer and F. Conraths, 2005. Oocysts of *Neospora caninum, Hammondia heydorni, Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. **Int. J. Parasitol**. 1525– 1537.

_____, M. Peters, R. Wurm, A. Barwald and F. J. Conraths. 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analyzed by serological techniques. **Vet. Parasitol.** 80:87-98.

Schatzberg, S.J., N.J. Haley, S.C. Barr, A. De Lahunta, N. Olby, K. Munana and N.J.H. Sharp. 2003. Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. **Am. J. Vet. Res**. 64:1507–1513.

Serrano, E., I. Ferre, K. Osoro, G. Aduriz, A. Mateos-Sanz, A. Martínez, R. Atxaerandio, C. O. Hidalgo and L. M. Ortega-Mora. 2006. Intrauterine *Neospora caninum* inoculation of heifers. **Vet. Parasitol.** 135:197-203.

Serrano-Martinez, E., I. Ferre, K. Osoro, G. Aduriz, R. A. Mota, A. Martínez, I. del-Pozo, C. O. Hidalgo and L. M. Ortega-Mora. 2007. Intrauterine *Neospora caninum* inoculation of heifers and cows using contaminated semen with different numbers of tachyzoites. **Theriogenology**. 67:729-737.

Slapeta, J.R., D. Modry, I. Kyselova, R. Horejs, J. Lukes and B. Koudela. 2002. Dog shedding oocysts of *Neospora caninum*: PCR diagnosis and molecular phylogenetic approach. **Vet. Parasitol**. 109:157–167.

Speer, C.A. and J,P. Dubey. 1989. Ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum*. **J. Protozool**. 36: 458-463.

_____, J.P. Dubey, M.M. McAllister and J.A. Blixt. 1999. Comparative ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum* and *Toxoplasma gondii*. **Int. J. Parasitol.** 29:1509-1519.

Suteeraparp, P., S. Pholpark, M. Pholpark, A. Charoenchai, T. Chompoochan, I. Yamane and Y. Kashiwazaki. 1999. Seroprevalence of antibodies to *Neospora caninum* and associated abortion in dairy cattle from central Thailand. **Vet. Parasitol**. 86:49–57.

Texas Department of State Health Services, 2010. **Immunofluorescence Procedures**. Available Source: http://www.dshs.state.tx.us/lab/serology_ifa.shtm, October 30, 2012.

The Secretariat of the Senate. 2002. **Relationship with the Thailand - Cambodia. (in Thai)** Available Source: http://www.senate.go.th/senate/report_ detail.php?report_id=10, October 1, 2012

Thornton, R.N., E.J. Thompson and J.P. Dubey. 1991. *Neospora* abortion in New Zealand cattle. **N.Z. Vet. J.** 39:129-133.

Thrusfield, M., C. Ortega,I. de Blas, J.P. Noordhuizen and K. Frankena. 2001. WIN EPISCOPE 2.0, Improved epidemiological software for WIN EPISCOPE 2.0, Improved epidemiological software for veterinary medicine. **Vet. Rec.** 148: 567-572.

Thurmond, M.C. and S. K. Hietala. 1996. Culling associated with *Neospora caninum* infection in dairy cows. **Am. J. Vet. Res**. 57:1559–1562.

Thurmond, M.C. and S. K. Hietala. 1997. Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. **Am. J. Vet. Res**. 58:1381–1385.

Tiwari, A., J.A. VanLeeuwen, I.R. Dohoo, H. Stryhn, G.P. Keefe and J.P. Haddad. 2005. Effects of seropositivity for bovine leukemia virus, bovine viral diarrhoea virus, *Mycobacterium avium* subspecies paratuberculosis, and *Neospora caninum* on culling in dairy cattle in four Canadian provinces. **Vet. Microbiol**. 109:147–158.

Trees, A.J., F. Guy, B.J. Tennant, A.H. Balfour and J.P. Dubey. 1993. Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. **Vet. Rec.** 132:125-126.

_____, M.M. McAllister, C.S. Guy, J.W. McGarry, R.F. Smith and D.J.L. Williams. 2002. *Neospora caninum*: oocyst challenge of pregnant cows. **Vet. Parasitol.** 109:147-154.

University of Liverpool. 2013. ***Neospora caninum*.** Available Source: http://www.testapet.com/test/Neospora.htm, January 29, 2013

van Maanen, C., W. Wouda, G. Schares, D. von Blumroder, F.J. Conraths, R. Norton, D.J.L. Williams, I. Esteban-Redondo, E.A. Innes, J.G. Mattsson, C. Bjorkman, A. Fernandez-Garcia, L.M. Ortega-Mora, N. Muller, H. Sager and A. Hemphill. 2004. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. **Vet. Parasitol**. 126:351–364.

von Blumroder, D., R. Stambusch, R. Labohm, W. Klawonn, K. Drager, W. Fasen, F.J. Conraths and G. Schares. 2006. Potential risk factors for the serological detection of Neospora caninum infections in cattle in Rhineland- Palatinate (Germany). **Tierarztl. Prax.** 34:141–147.

Waldner, C.L., E.D. Janzen and C.S. Ribble. 1998. Determination of the association between *Neospora caninum* infection and reproductive performance in beef herds. **J. Am. Vet. Med. Assoc**. 213:685–690.

Wiengcharoen, J., C. Nakthong, J. Mitchaothai, R. Udonsom and Y. Sukthana. 2012. Toxoplasmosis and Neosporosis among beef cattle slaughtered for food in Western Thailand. **Southeast Asian J. Trop. Med. Public Health.** 43: 1087-1093.

Wiengcharoen, J., C. Thuchadaporn, P. Suvarin and J. Nattaya. 2010. High Prevalence of Antibodies to *Neospora caninum* in Water Buffaloes (*Bubalus Bubalis*) from a local farm in Chachoengsao province. **In Proceedings of the 4th MUT Veterinary Annual Conference.** Mahanakorn University of Technology, Bangkok, Thailand (Nov 26, 2010)

Wouda, W., T. Dijkstra, A.M.H. Kramer, C. van Maanen and J.M.A. Brinkhof. 1999. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. **Int. J. Parasitol**. 29:1677– 1682.

_____, A.R. Moen and Y.H. Schukken.1998. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. **Theriogenology.** 49:1311-1316.

_____, _____, I.J.R. Visser and F. van Knapen. 1997. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. **J. Vet. Diagn. Invest.** 9:180– 185.

Yu, J., Z. Xia, Q. Liu, J. Liu, J. Ding and W. Zhang. 2007. Seroepidemiology of *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes (*Bubalus bubalis*) in the People's Republic of China. **Vet. Parasitol.** 143:79–85.

Zhai, Y.Q., J.P. Zhao, X. Q. Zhu, L. Li and C.R. Wang. 2007. Research Advances in the Diagnosis of Cattle Neosporosis. **J.Anim. Vet. Adv**. 6:1377-1387.

APPENDICES

Appendix A

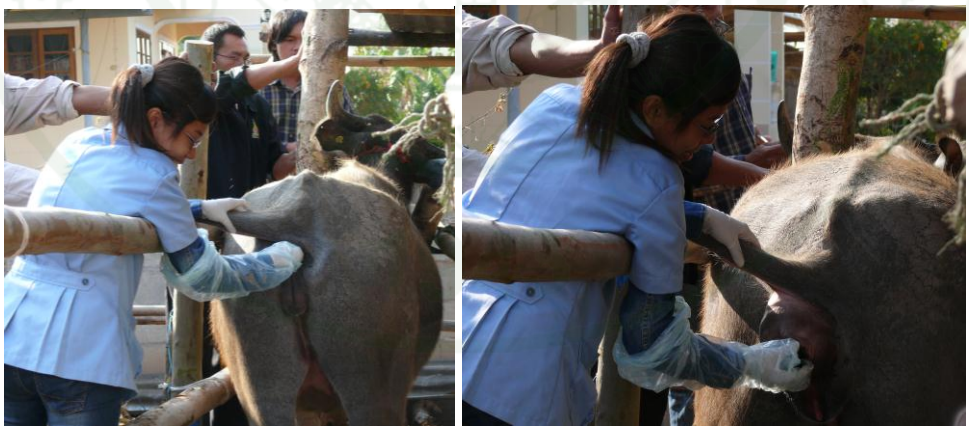
The buffalo husbandy and the sample collection in each province in this study

Appendix Figure A1 The buffalo husbandy in Buri Ram

Appendix Figure A2 The buffalo husbandy in Si Sa Ket

Appendix Figure A3 The buffalo husbandy in Surin

Appendix Figure A4 The blood sample collection



Appendix Figure A5 The fecal sample collection

Appendix B

The standard method

1. Recovering cell

1. Thaw African green monkey kidney (Vero cell) from liquid N_2 at room temperature.
2. Move Vero cell to centrifugal tube (10 ml) and add 9 ml of MEM.
3. Centrifuge at 1,448 G at 4°c for 5 minutes.
4. Remove 9 ml of supernatant.
5. Add 1 ml of MEM in the centrifugal tube and mix it gently by pipette.
6. Transfer cell in MEM to new flask (1 ml for 25 cm^3) and incubate at 37°c in 5% CO_2 incubator with 70 – 80% of humidity.
7. Check cell in the flask every 3 days.

2. Splitting cell

1. Discard old MEM from the flask with Vero cell.
2. Wash the flask with PBS, mix it gently, and discard PBS.
3. Add 1 ml of trypsin to trypsonize cell on the base of flask.
4. Incubate at 37°c for 15 minutes.
5. Check peeling cell in the flask using the invert microscope.
6. Add 5 ml of MEM into the flask and mix it gently.
7. Transfer 1 ml of cell in MEM from old flask to new flask.
8. Add 4 ml of MEM to new flask and mix it gently.
9. Incubate at 37°c in 5% CO_2 incubator with 70 – 80% of humidity.

3. Infecting cell

1. Thaw *N. caninum* from liquid N_2 at room temperature.
2. Move *N. caninum* to a centrifugal tube (10 ml) and add 9 ml of MEM.
3. Centrifuge at 1,448 G at 4°c for 5 minutes.
4. Remove 9 ml of supernatant.
5. Transfer *N. caninum* to the flask with Vero cell in new MEM.
6. Check *N. caninum* in Vero cell every 2 days.

7. Wash the flask with PBS and add MEM to flask if there are dead cells.

4. Harvesting cell or cell and parasite

1. Check whether the number of cell in a flask is enough or not.
2. Scrape off cell with cell scraper.
3. Remove the culture to a centrifugal tube (10 ml).
4. Centrifuge at 1,448 G at 4°c for 5 minutes.
5. Remove the supernatant.
6. Add 5 ml of PBS to the centrifugal tube and mix it gently.
7. Centrifuge at 1,448 G at 4°c for 5 minutes.
8. Remove the supernatant.
9. Add 1 ml of freezing media and mix it gently.
10. Remove cell and media to a cryotube.
11. Keep in -20°c overnight.
12. Move it to liquid N_2 for storage.

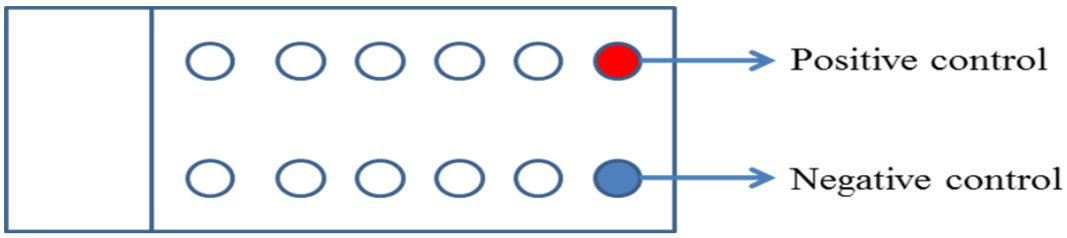
5. Purification of *N. caninum*

1. Check whether the number of parasite in a flask is enough or not.
2. Scrape off parasite with cell scraper.
3. Suck the culture and release it back into the flask 3 times using gauge 25 needle with 5 ml-hypodermic syringe following by bottle 3 times using gauge 27-needle with 5 ml-hypodermic syringe.
4. After breaking the cell membrane, aspirate the parasite into a syringe again and fix the 5µm-millipore filter and filter out.
5. Centrifuge at 1,448 G for 5 minutes at 4°c.
6. Remove the supernatant and add 10 ml PBS and centrifuge at 1,448 G for 5 minutes at 4°c.
7. Remove the supernatant.
8. Dilute with PBS and transfer it into 1.5 ml tube and label.

9. Suck the parasites and release it back into 1.5 ml tube 3 times using gauge 27-needle with 5 ml-hypodermiic syringe.
10. Count the parasite cells using hemocytometer.
11. Dilute it as1x10^4 parasite/ml by PBS.
12. Add 10 µl each well and dry it at room temperature for IFAT slide.
13. Transfer the parasite to a cryotube, add 1 ml of freezing media and mix it gently.
14. Keep in -20°c overnight.
15. Move it to liquid N_2 for storage.

6. Protocol for IFAT (*N. caninum*)

1. Layer 10 µl of a whole tachyzoite preparation on to each 4-mm well of Teflon-coated antigen slides and allow to dry at room temperature.
2. Fix in acetone for 30 minutes and lets it dry.
3. Carry out 10 minutes washes in PBS, pH 7.4.
4. Add 10 µl of diluted test serum as 1:100 (diluted in PBS) to each well. Ensure that positive and negative control sera are included in each test. Incubate for 30 minutes at 37°c.
5. Carry out 15 minutes washes in PBS.
6. Add 10 µl of an appropriate dilution of rabbit-anti bovine IgG conjugated to fluorescein isothiocyanate to each well and incubate at 37°c for 30 minutes.
7. Carry out 15 minutes washes in PBS.
8. Mount the slides under cover slips with buffered glycerol (nine part PBS/one part glycerol).
9. Examine using a fluorescence microscope, fitted with X20 or X40 objective lens.



Appendix C

Chemical reagents and substances

1. PBS X10 (Stock solution)

Na_2HPO_4 (anhydrous)	58.64	g
NaH_2PO_4 (anhydrous)	9.60	g
NaCl (anhydrous)	438.75	g
Distilled water	5	liters

2. PBS pH 7.4 (Working solution)

PBS X10	100	ml
Distilled water	900	ml

Shake 20 minutes and set the pH at 7.4 by 1N HCl

3. Minimum Essential Medium solution (MEM)

$NaHCO_3$	2.20	g
MEM power (Gibco^{TM})	1	pack
Distilled water	900	ml

Filtrated by Millipore filter in Erlenmeyer flask with vacuum pump and keep in 4°c

4. L – glutamine

L – glutamine (Biochrom AG)

5. Penicillin and streptomycin

Pen Strep (Gibco^{TM})

6. 0.25% Trypsin with EDTA

0.25% Trypsin – EDTA (Gibco^{TM})

7. Fetal Bovine Serum (FBS)

FBS (PAA)

Inactivated at 56°c for 30 minutes

8. Dimethyl sulfoxide (DMSO)

DMSO (Merck)

9. Growth media (Containing 10% FBS)

FBS	5 ml
L – glutamine	500 µl
Pen – strep	100 µl
MEM	45 ml

10. Maintenance media (Containing 2% FBS)

FBS	1 ml
L – glutamine	500 µl
Pen – strep	100 µl
MEM	49 ml

11. Freezing media

MEM	8 ml
FBS	1 ml
DMSO	1 ml

CURRICULUM VITAE

NAME **:** Miss Chanya Kengradomkij

BIRTH DATE **:** October 22, 1982

BIRTH PLACE **:** Bangkok, Thailand

EDUCATION **:**

YEAR	**INSTITUTE**	**DEGREE**
2005	Kasetsart Univ.	B. Sc. (Veterinary Technology)

POSITION/TITLE **:** -

WORK PLACE **:** Faculty of Veterinary Medicine, Kasetsart University

SCHOLARSHIP/AWARDS **:** -