CHAPTER III
MATERIALS AND METHODS

1. Investigation of the prevalence of *N. caninum* infection in swamp buffaloes in Northeast, Thailand

1.1 Animals

Investigated swamp buffaloes were raised in 5 provinces in Northeast Thailand which are Khon Kaen, Maha Sarakham, Nong Khai, Sakon Nakhon and Udon Thani (Figure 8). In 2009, there were approximately 29,000, 44,000, 33,000, 76,000 and 60,000 and swamp buffaloes in these five provinces, respectively (DLD, 2009).

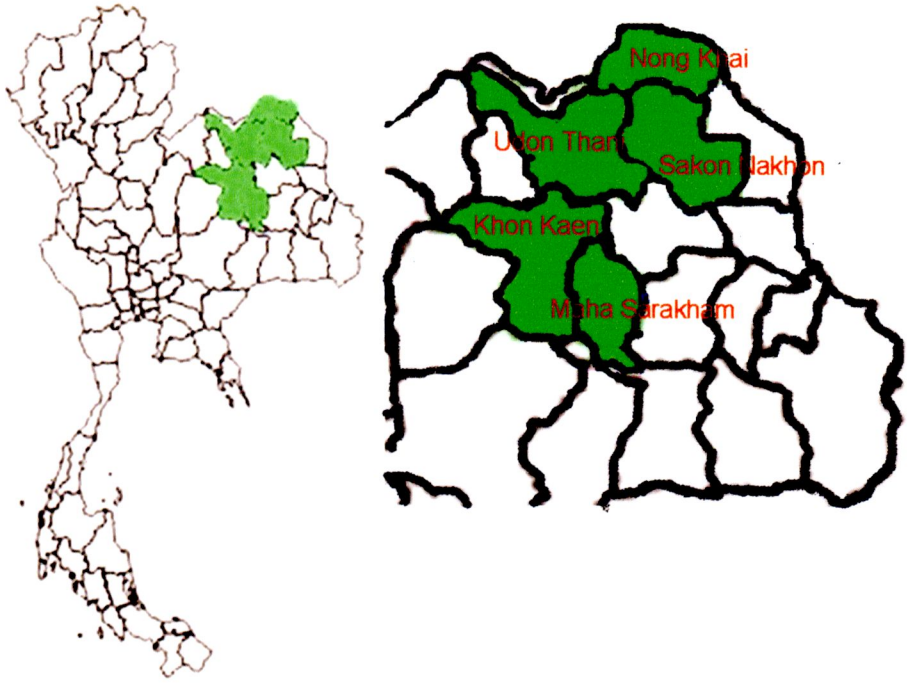

Figure 8 Map of Northeast Thailand and five provinces from which blood samples of swamp buffaloes were collected.

1.2 Sample collection and storage

To investigate the prevalence of antibodies to *N. caninum* in swamp buffaloes, a

cross-sectional study was carried out. From January to the end of July 2009, serum samples from 532 swamp buffaloes were collected in those five provinces. In detail, 187, 60, 31, 78 and 176 swamp buffaloes were sampled in 1) Phu Wiang district, Khon Kaen province, 2) Kosum Phisai district, Maha Sarakham province, 3) Tabor district, Nong Khai province, 4) Kudbak district, Sakon Nakhon province and 5) Muang district, Udon Thani province, respectively. The swamp buffaloes in Udon Thani were gathered from several places within province, and from other provinces in a charitable project.

Ten ml of blood was drawn from the jugular vein, then dispensed into the collection tubes and kept in the ambient temperature. All the samples were transferred to the laboratory within 4-6 hours for further analysis.

Age of swamp buffaloes were determined based on their permanent teeth. Buffaloes had no, one, two, three or four pairs of permanent teeth were judged to be less than 2, from 2-3, from 3-3.5, from 3.5-4.5 or more than 4.5 years old, respectively (Bragulla et al., 2007; Dyce et al., 1996; Pace and Wakeman, 1983; Smith, 2002).

Clot blood was centrifugated for 15 minutes at 1000 x *g*. Sera were dispensed into micro-tubes and kept at -20^{0}C until analysis

1.3 *N. caninum* antibodies demonstration

Presence of specific antibodies in sera samples was analyzed by *N. caninum* iscom ELISA (SVANOVIR Neospora-Ab iscom ELISA, Svanova Biotech AB, Sweden). The test was used to demonstrate *N. caninum* antibodies in cattle and buffalo samples (Bjorkman et al., 1997; Huong et al., 1998). Sera were diluted at 1:100 in PBS-Tween before analysis. Positive and negative controls were included in every plate in duplicates. The optical density (OD) was measured at 450 nm. OD values of all the samples were correlated to the OD of the positive control to calculate percent positivity. Samples were judged positive if the percent positivity was more than 20.

1.4 Statistical analysis

Comparisons of prevalences of *N. caninum* infection in different groups of swamp buffaloes (location, age, gender) were conducted by using Chi-square tests at 95%

confident interval (SPSS version 17).

2. Study of effects of *N. caninum* infection on the fertility of artificially inseminated swamp buffaloes

To evaluate the influence of *N. caninum* infection on the fertility of swamp buffaloes, 115 female buffaloes in Maha Sarakham, Nong Khai and Sakon Nakhon were synchronized for estrus. Sera samples were also collected during synchronized program for examination of the *N. caninum* antibodies.

2.1 Estrus synchronization and pregnancy diagnosis

The synchronization program in female swamp buffaloes was carried out by Khon Kaen Artificial Insemination Research Center, Department of Livestock Development. Female swamp buffaloes were induced synchronous estrus by applying 3 hormone programs as following.

Program 1: Progestagen, $PGF_{2\alpha}$ and GnRH were applied.

On the first day of synchronization, swamp buffaloes were injected GnRH, and implanted progestagen (Cresta) in their necks and ears, respectively. A subcutaneous injection of estradiol valerate was also performed right after the implantation. On the day 7^{th}, $PGF_{2\alpha}$ was applied to the buffaloes while Cresta was removed. The second injection of GnRH was conducted on the day 9^{th}. Buffaloes were then artificially inseminated twice on the day 10^{th} and 11^{th}.

Program 2: $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus GnRH were used in this program. The details of procedures were described below.

1. $PGF_{2\alpha}$ was injected twice on the day 1^{st} and 11^{th}. GnRH was applied on the day 13^{th} and artificial insemination was conducted twice on the day 14^{th} and 15^{th}.
2. Buffaloes were artificially inseminated twice on the day 14^{th} and 15^{th} after two injections of $PGF_{2\alpha}$ on the day 1^{st} and 11^{th}.

Program 3. This proram used GnRH and $PGF_{2\alpha}$ to manipulate the follicular phase in the ovaries of the buffaloes.

GnRH was injected twice on the day 1^{st} and 9^{th} of the program, one injection of $PGF_{2\alpha}$ was on the day 7^{th}. Artificial insemination was done twice on the day 10^{th} and 11^{th}.

GnRH (Receptal) and $PGF_{2\alpha}$ (Estrumate) were used at the doses of 22 μg and 500 μg, respectively. Pregnancy diagnosis was conducted by ultrasound screening at either day 45 or 60 post-artificial insemination.

2.2 Statistical analysis

Comparison of conception rate in different categories of swamp buffaloes (hormone programs, provinces and sero-status) was done by using Chi-square test at 95% confident interval. Logistic regression measuring the association between conception rate and other factors including serostatus, hormone programs and location was also conducted (SPSS version 17).

3. Prevalence of *N. caninum* infection in swamp buffaloes and beef cattle reared in the same area

In this experiment, 187 swamp buffaloes and 78 beef cattle in 30 and 19 farms in Phu Wiang district, Khon Kaen province were sampled. All the methods of blood collection, sera extraction and antibodies demonstration were similarly described in the first study.

The comparison of prevalence of *N. caninum* infection between swamp buffaloes and beef cattle, and among beef cattle groups (age, gender) was performed by using Chi-square test at 95% confident interval (SPSS version 17).

4. Demonstration of the *N. caninum* DNA in the swamp buffalo whole blood

Eighty-one swamp buffaloes from 9 farms those had at least one positive buffalo in each in Phu Wiang district, Khon Kaen province were sampled for both whole blood and serum. Nested-PCR was applied to demonstrate *N. caninum* DNA in the buffalo whole blood while iscom ELISA was used to examine the presence of *N. caninum* antibodies.

4.1 DNA extraction for PCR

Fifty and thirty-one buffalo whole blood samples were extracted by using DNeasy Blood & Tissue Kit and TRIZOL-LS Reagent, respectively. The extraction of

DNA by DNeasy Blood & Tissue Kit utilized 100 µl of anti-coagulated blood while that of TRIZOL LS Reagent used 250 µl. After the extraction, DNA template was stored at -20^{0}C until analysis.

4.2 DNA amplification

The condition of amplification of *N. caninum* DNA using Nested-PCR was performed according to Yao et al. (2009).

Primary amplification: Total volume of one reaction was 50 µl. Each reaction contained 5 µl of DNA template, 5 µl of 10 x CoralLoad PCR, 10 µl of 5 x Q-solution, 1 µl of d-NTP mix (100 µM of each d-NTP), 1.5 µl of each primer Np21$^+$ and Np6$^+$ (0.5 µM of each), 0.5 µl of Taq DNA polymerase (2.5 units) and 25.5 µl of distilled water. The PCR program started with an initial denaturation at 94^{0}C for 5 minutes followed by 35 cycles in which denaturation was at 94^{0}C for 30 seconds, annealing at 63^{0}C for 20 seconds and extension at 72^{0}C for 30 second. The final extension was set at 72^{0}C for 10 minutes. This PCR step was to amplify a fragment of 328 bp of the Nc5 gene.

Secondary amplification: Each reaction contained 1 µl of primary amplification products diluted at 1:10, 5 µl of 10 x CoralLoad PCR, 10 µl of 5 x Q-solution, 1 µl of d-NTP mix (100 µM of each d-NTP), 1.5 µl of each primer Np9 and Np10 (0.5 µM of each), 0.5 µl of Taq DNA polymerase (2.5 units) and 29.5 µl of distilled water. The condition of the reaction was the same as the primary amplification. This PCR step was to amplify a fragment of 224 bp of the Nc5 gene.

4.3 Electrophoresis and gene bands detection

Solid 0.1% agarose in 0.5 x TBE was melt down in a microwave oven until the solution looked completely clear. The solution was then cooled down and poured into a chamber to fix the agarose. At one end of the chamber, a comb was used to make wells into which PCR products would be dispensed. When the agarose turned back into solid, the comb was carefully removed and the chamber was placed into the electrophoresis machine which contains 0.5% TBE solution. DNA 100 bp marker and PCR products were dispensed into corresponding wells then the machine was set to run at 100 V for 60

minutes.

After electrophoresis, the agarose was stained in 1% ethidium bromide solution for 10 minutes before washed in container under mild running water for 10 minutes. The agarose was finally placed onto the ultraviolet machine to read the bands of DNA. Samples were considered positive if there were bands of DNA fragments of either 328 bp or 224 bp.