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APPENDICES

Appendix 1 Collection of samples





Appendix 2 ELISA technique

Materials for iscom ELISA test (Svanova Biotech AB, Sweden)

- Sera of swamp buffaloes
- *Neospora caninum* positive control serum - 0.05% merthiolate
- *Neospora caninum* negative control serum – 0.05 % merthiolate
- Neospora antigen coated microtiter strips
- Lyophilized HRP conjugate (horseradish peroxidase conjugated anti-bovine IgG monoclonal antibodies)
- PBS –Tween solution 20 x concentrate
- Substrate solution (tetramethyl-benzidine in substrate buffer containing H_2O_2)
- Stop solution- contains sulphuric acid

- Multi-channel micro pipette
- Plates for sera dilution
- Single channel micro pipette
- Disposable pipette tips
- Distilled water
- Containers: 1 liter for PBS-Tween, triangular bottomed containers for PBS-Tween, conjugate, substrate, stop solution
- Micro-plate photometer, 450 nm filter
- Racks, permanent pen, tissue, gloves, tip container

Iscom ELISA procedures

Step 1: All the reagents were equilibrated to room temperature before use.

Step 2: Preparation of PBS-Tween buffer: Dilute 25 ml of PBS-Tween solution 20 x concentrate in 475 ml distilled water and mix thoroughly.

Step 3: Dilution of controls and samples: Ninety-five μ l PBS-Tween buffer was dispensed into each well of a plate then 5 μ l of controls or samples was added and mixed thoroughly. Twenty μ l of this solution was aspirated and put into an iscom ELISA plate. Eighty μ l of PBS-Tween buffer was then added into each well of the iscom ELISA plate. Controls were tested in duplicates while samples were analyzed either in duplicates or singles. The plate was covered and shaken thoroughly and incubated for 1 hour at 37°C.

Step 4: The plate was rinsed three times with PBS-Tween buffer. At each rinse cycle, all the wells were filled with 200 μ l of PBS-Tween buffer and taped hard to remove the remains of fluids.

Step 5: One hundred μ l of conjugate was added into each well and the plate was covered and incubated for 1 hour at 37°C.

Step 6: The step 4 was then repeated.

Step 7: One hundred μ l of substrate was added into each well. The plate was covered and incubated at room temperature for 10 minutes. Timing was begun when the first well was filled.

Step 8: The reaction was stopped by adding 50 µl of stop solution into each well in the same order as the substrate solution.

Step 9: The optical density (OD) was measured in a micro-plate photometer. This measurement was conducted within 15 minutes after the addition of the stop solution.

Calculation of percent positivity values (PP)

$$PP = \frac{\text{Mean OD value (Sample or Negative control)}}{\text{Mean OD value (Positive control)}} \times 100$$

Interpretation:

PP < 20: Negative

PP ≥ 20: Positive

Pictures illustrating ELISA technique



Blood samples



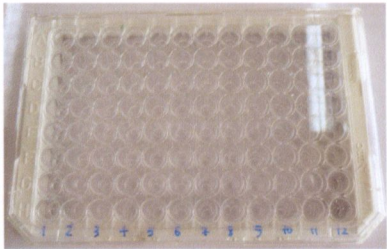
Centrifugation



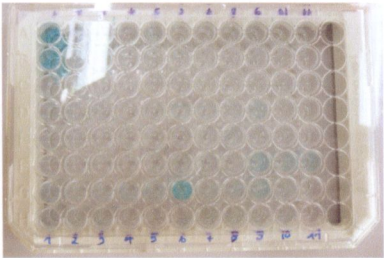
Sera



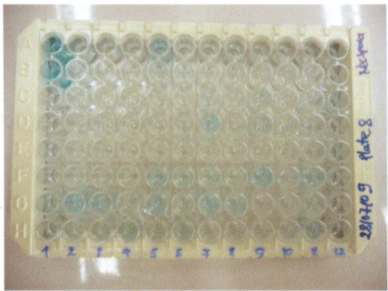
Store sera in freezer at -20°C



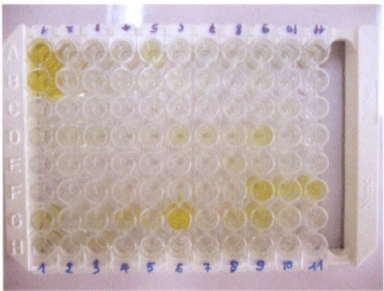
Neospora- ELISA plate



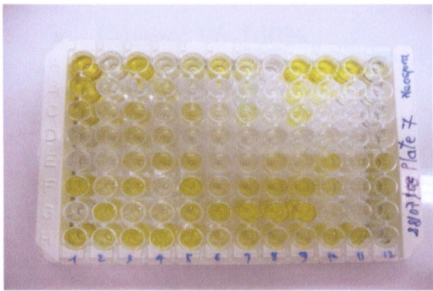
ELISA plate after adding substrate



ELISA plate after adding substrate



ELISA plate after adding stop solution



ELISA plate after adding stop solution



OD values reading

1.371	0.108	0.094	0.106	0.198	0.101	0.092	0.108	0.123	0.088	0.15	0.001
1.336	0.086	0.112	0.102	0.101	0.114	0.129	0.074	0.129	0.088	0.088	0
0.076	0.076	0.067	0.083	0.069	0.088	0.067	0.055	0.09	0.111	0.082	0
0.145	0.148	0.133	0.106	0.123	0.186	0.177	0.136	0.245	0.102	0.117	0
0.09	0.087	0.086	0.08	0.082	0.088	0.055	0.152	0.096	0.096	0.102	0
0.07	0.076	0.106	0.105	0.069	0.085	0.09	0.093	0.381	0.288	0.388	0
0.241	0.118	0.133	0.216	0.159	0.123	0.113	0.209	0.311	0.144	0.116	0
0.162	0.107	0.187	0.074	0.08	0.081	0.075	0.131	0.059	0.112	0.074	0

1.475	0.119	1.005	0.151	0.257	0.392	0.264	0.082	0.838	1.245	0.807	0.17
1.404	0.105	0.098	0.228	0.075	0.083	0.145	0.082	0.311	0.223	0.137	0.098
0.312	0.189	0.296	0.123	0.283	0.147	0.231	0.104	0.31	0.092	0.079	0.103
0.137	0.171	0.218	0.199	0.1	0.14	0.102	0.123	0.095	0.123	0.109	0.142
0.086	0.104	0.221	0.269	0.264	0.118	0.134	0.224	0.168	0.361	0.156	0.303
0.713	0.21	0.362	0.094	0.688	0.174	0.27	0.317	0.436	0.285	0.128	0.252
0.107	0.415	0.155	0.161	0.291	0.516	0.82	0.809	1.177	0.128	0.127	0.189
0.478	0.296	0.472	0.61	0.517	0.215	0.275	0.225	0.202	0.316	0.401	0.14

OD values

Appendix 3 Nested-PCR

Materials for DNA extraction by DNeasy kit (QIAGEN Group)

- DNeasy Mini Spin Columns in 2 ml collection tubes
- Collection tubes (2 ml)
- Buffer AL
- Buffer AW1 (concentrate).
- Buffer AW2 (concentrate)
- Buffer AE
- Proteinase K
- Ethanol 96-100%
- PBS solution
- 99% ethanol
- Micropipettes (20-200 µl)
- Ice box with ice
- Vortexer
- Micro-centrifuge
- Heater
- Collection tube containers

- Permanent pen

Before use, 99% ethanol was added to the AW1 and AW2 solutions to obtain working reagents.

DNA extraction by DNeasy Blood & Tissue Kit

Step 1: 100 μ l of anti-coagulated blood were added into 1.5 ml micro-centrifuge tube which contained 20 μ l of Proteinase K. Adjust the volume to 220 μ l with PBS.

Step 2: Add 200 μ l of buffer AL. Mix thoroughly by vortexing, and incubate at 56°C for 10 minutes.

Step 3: Add 200 μ l of 96% ethanol to each sample, and mix thoroughly by vortexing.

Step 4: Pipett the mixture from the step 3 into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at $\geq 6,000 \times g$ (8,000 rpm) for one minute. Discard flow-through and collection tube.

Step 5: Place the DNeasy Mini spin column into a new 2 ml collection tube, add 500 μ l of AW1, and centrifuge for 1 minute at $\geq 6,000 \times g$ (8,000 rpm). Discard flow-through and collection tube.

Step 6: Place the DNeasy Mini spin column into a new 2 ml collection tube, add 500 μ l AW2, and centrifuge for 3 minute at $\geq 2,0000 \times g$ (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

Step 7: Place the DNeasy Mini spin column into a clean 1.5 ml micro-centrifuge tube, and pipet 200 μ l of buffer AE directly into the DNeasy membrane. Incubate at room temperature for 1 minute, and then centrifuge at $\geq 6,000 \times g$ (8,000 rpm) for 1 minute to elute the DNA.

The DNA template was then kept at -20°C until used in a nested-PCR.

Materials used for DNA extraction by TRIZOL

- Trizol LS reagent
- Chloroform
- Ethanol

- 0.1 M Sodium citrate in 10% ethanol
- 75% ethanol
- 8m M NaOH
- Centrifuge
- Pointed bottomed plastic tubes

DNA extraction by TRIZOL LS Reagent

Step 1-Homogenization: Dispense 0.25 ml of blood sample into each plastic tube followed by 0.75 ml of Trizol LS reagent. Incubate for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

Step 2-Phase separation: Add 0.2 ml of chloroform. Shake vigorously by hand for 15 seconds then incubate at room temperature for 15 minutes. Centrifuge at 12000 x g for 15 minutes at 4⁰C. Following this step mixture separates into 3 phases, i.e. a lower red, phenol-chloroform phase, an interphase, and an colorless upper aqueous phase. DNA is in the interphase and organic-phase with ethanol.

Step 3-DNA precipitation: Remove aqueous phase. Add 0.3 ml of 100% ethanol, mix by inversion. Store samples at room temperature for 2 minutes, and sediment DNA by centrifugation at 2000 x g for 5 minutes at 4⁰C.

Step 4-DNA wash: Remove the phenol-ethanol supernatant. Wash DNA pellet twice in a solution containing 0.1 M sodium citrate in 10% ethanol. At each wash, use 1 ml of this solution and store the DNA pellet at room temperature for 30 minutes and centrifuge at 2000 x g for 5 minutes at 4⁰C. Following these two washes, suspend pellet DNA in 2 ml of 75% ethanol. Store for 10 minutes at room temperature, and centrifuge at 2000 x g for 5 minutes at 4⁰C.

Step 5-Redissolving the DNA: Dry the pellet DNA for 5 minutes under vacuum and dissolve in 0.3 ml of 8 mM NaOH. Centrifuge at 5000 x g for 10 minutes at room temperature to remove insoluble materials. Transfer the supernatant containing DNA in to centrifuge tube and store at -20⁰C.

Materials used for Nested-PCR (QIAGEN Group)

- 10 x CoralLoad PCR
- 5 x Q-solution
- d NTP mix
- Primer Np21+ (5'CCCAGTGCGTCCAATCCTGTAAC3')
- Primer Np6+ (5'CTCGCAGTCAACCTACGTCTTCT3')
- Primer Np9 (5'GTTGCTCTGCTGACGTGTCGTTG3')
- Primer Np10 (5'CTCAACACA GAACACTGAACTCTCG3')
- Taq DNA polymerase
- Distilled water
- Template DNA
- Heater
- Micro-centrifuge
- Micropipettes (0.1-200 µl)
- PCR tubes
- Containers for PCR tubes
- Ice box
- Portable chill box to keep Taq DNA polymerase at -20⁰C
- Permanent pen
- Micro-centrifuge tubes for Master mix.

Materials used for electrophoresis

- TBE buffer
- 0.1% agarose 0.5 x TBE
- Microwave oven
- Chamber for agarose fixation
- Electrophoresis machine
- 1% ethidium bromide solution
- UV machine
- Camera

Pictures illustrating the PCR technique



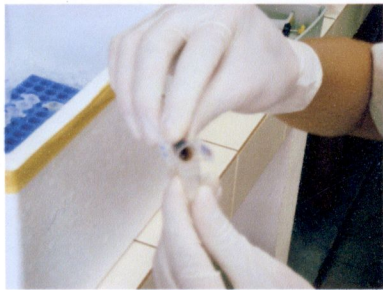
Blood samples



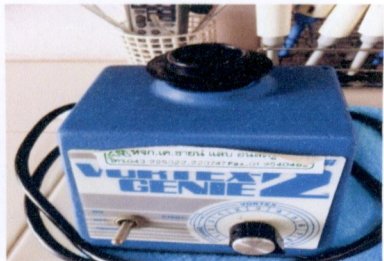
DNeasy extraction kit



Extracting DNA



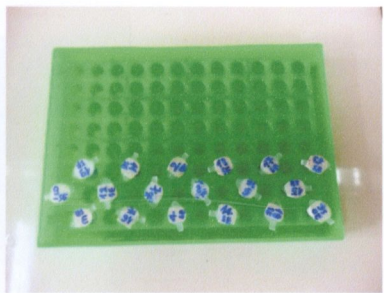
Heater and micro-centrifuge used in DNA extraction



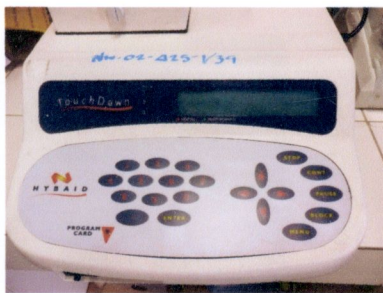
Vortexer



Nested-PCR reagents



PCR- tubes



Thermocycler

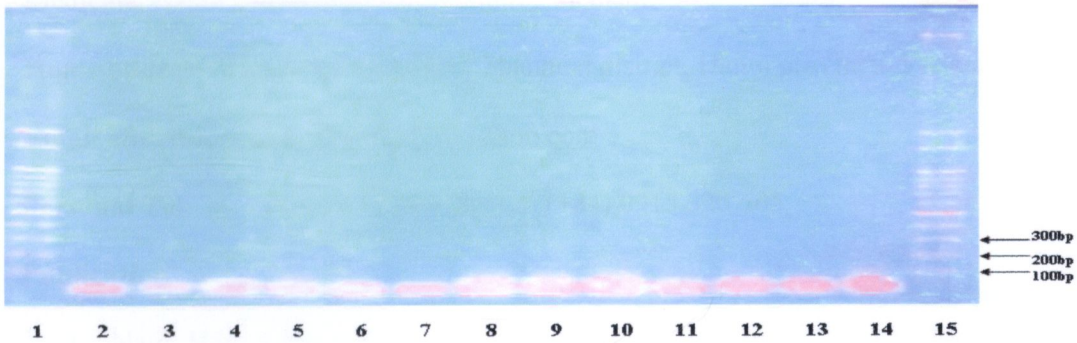


Electrophoresis





Electrophoresis



Lane 1 and 15: ladder 100 bp, lane 2: negative control, lanes 3-4: seropositive samples, lanes 5-14 seronegative samples.

Viewing DNA band under ultraviolet light

CURRICULUM VITAE



Full name: Hoainam Nguyen

Date of birth: June 26th 1982

Place of birth: Thanhmai commune, Thanhhoai district, Hatay province

Bachelor degree: The author obtained Bachelor degree of Doctor of Veterinary Medicine in 2005 from Hanoi University of Agriculture, Vietnam.

Place of work: Department of Animal Surgery and Reproduction, Faculty of Veterinary Medicine, Hanoi University of Agriculture, Vietnam.

Home address: Thanhmai commune, Thanhhoai district, Hanoi province, Vietnam.

Email address: hoainam26061982@yahoo.com

Personal Tel: (+66) 874389148; (+84) 0915 325 505

Publication

1. Nam, H.N., Chanlun, A., Aiumlamai, S. and Kanistanon, K. 2009. Prevalence of *Neospora caninum* infection in swamp buffaloes in northeast Thailand. In the Proceedings of 2nd FASAVA 2009, Bangkok, Thailand, 3-5th November, 2009. p 566-567. Poster presentation.

2. Nam, H.N., Chanlun, A., Kanistanon, K. and Aiumlamai, S. 2010. Survey of *Neospora caninum* infection in swamp buffaloes and beef cattle in Khon Kaen, Thailand. In the Proceedings of 11th KKU Veterinary Annual Conference, Khon Kaen, Thailand, 10-11th June, 2010. p 41-47. Oral and poster presentation.

