

***Trypanosoma evansi* AND *Neospora caninum* AMONG WATER BUFFALOES
(*Bubalus bubalis*) IN THE PHILIPPINES**

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ABSTRACT: The study determined the positivity rate of *Trypanosoma evansi* and *Neospora caninum* antibodies in water buffaloes in the province of Nueva Ecija, Philippines using Polymerase Chain Reaction (PCR) for *T. evansi* and competitive Enzyme-linked Immunosorbent Assay (cELISA) for *N. caninum* antibodies. A total of 100 whole blood and 100 serum samples were collected to test for *T. evansi* and *N. caninum*, respectively. *Rotat 1.2 VSG* gene was target using PCR for *T. evansi* detection. *Neospora caninum* antibody detection was done from the serum samples using cELISA test kit. Results revealed that the positivity rate of *T. evansi* in Nueva Ecija was 11% (11/100). The positive animals identified were from the municipalities of Muñoz (4/16; 25%), Sta. Rosa (3/13; 23.08%) and Talugtug (4/16; 25%). The seropositive rate of Nueva Ecija for *N. caninum* was 46% (46/100), seropositive animals were identified in Cabanatuan City, 57.14% (4/7); Science City of Muñoz, 43.14% (22/51); Sta. Rosa, 40% (4/10); Sto. Domingo, 50% (6/12); and Talugtug 50% (10/20). The seropositivity rate of *N. caninum* and the presence of *T. evansi* in Nueva Ecija may contribute to the cases of abortions in the province and further studies should be employed to confirm the association of these organisms to abortion cases on water buffaloes..

Keywords: Trypanosoma; Neospora; PCR; cELISA; water buffalo.

INTRODUCTION

Philippine water buffalo (*Bubalus bubalis*) is one of the most important livestock in many developing countries due to its adaptability to hot and humid tropical areas (Villanueva et al., 2016). It has a significant role in the agricultural economy of many developing countries such as providing meat and milk for nutrition and draught power for agriculture and transport (De Alwis et al., 1999). In the Philippines, the population of water buffalo is about 2.88 million heads (PSA, 2016), majority of the contributors are smallholder farmers who prefer water buffalo as a livestock animal over cattle for its ability to perform optimally under relatively adverse environmental conditions (De Alwis et al., 1999) and considered more resistant to several bovine tropical diseases (Warriach et al., 2015). Despite these merits, water buffaloes are not excused from infectious agents that cause abortion leading to a compromised reproductive efficiency.

Abortion is the delivery of an immature fetus, either dead or alive before the expected parturition time as a result of failure of the mechanisms that control pregnancy (Shaapan, 2016). Both noninfectious (nutritional, physical, toxic and chemical) and infectious (viral, bacterial, fungal and protozoal) etiologies can result in pregnancy loss. Infectious agents are perhaps the most frequently thought of cause of abortions in human and domestic animals (Pretzer, 2008) and these includes some abortigenic protozoans. The most common protozoal diseases responsible for abortion incidence in domestic animals are neosporosis (Dubey and Lindsay, 1996), trypanosomiasis (Lun et al., 1993), sarcocystosis (Shaapan, 2016), toxoplasmosis (Pretzer, 2008) and trichomoniasis (Sanjrani et al., 2013). However, at present, there is limited epidemiological information and

data regarding abortion in water buffaloes in Nueva Ecija leading to insufficient consideration given to the implementation of systematic control measures against major abortifacient infectious agents in livestock (Konnai et al., 2008). Few studies have been carried out on the identification of agents causing abortion in livestock animals, however, such studies focus mainly on cattle (Bombio et al., 2010, Konnai et al., 2008, Ochirkhuu et. al., 2015) and limited on water buffaloes.

The study on these protozoal agents of water buffaloes will give knowledge on the positivity rate of *T. evansi* and *N. caninum* antibodies in Nueva Ecija. This will give information about the possible presence of these parasites and can be used by future studies to confirm the agent itself especially *N. caninum* and to provide a better understanding on the economic impacts of these pathogens and a basis for control and preventive measures on the area.

MATERIALS AND METHODS

Sample collection

A total of 200 blood samples of apparently healthy water buffaloes were collected randomly in selected municipalities and cities in northern Philippines. These buffaloes were restrained in a chute and approximately 5 ml of blood samples were collected aseptically via jugular venipuncture or at the coccygeal vein. One hundred blood samples were collected in tubes with anticoagulant for *T. evansi* detection while the rest of the samples were placed in tubes without anticoagulant to easily separate the serum. Collected samples were transported immediately to the laboratory for processing.

DNA extraction and PCR for the detection of *T. evansi*

Whole blood samples with EDTA were extracted using the conventional DNA extraction method.

PCR reaction mixture was prepared in a master mix with a total volume of 12.5 µL containing: 2 µL *Taq* Buffer, 0.2 µL dNTP mixture, 0.8 µL MgCl₂, 0.05 µL *Taq* Polymerase, 0.25 µL of each primer, 1.5 µL of extracted DNA and 7.45 µL of DDW.

Specific primers used for the detection of *T. evansi* amplify an approximately 205 base pairs (bp) gene fragment of the *RoTat 1.2 VSG* gene (AF317914). The primer pair is as follows:

5'-GCGGGGTGTTTAAAGCAATA-3' and 5'-ATTAGTGCTGCGTGTGTTTCG-3'.

This primer pair targets the DNA region lacking homology to other known VSG genes. According to Claes *et al.* (2004) PCR amplification of the *RoTat 1.2 VSG* gene is a specific marker for *T. evansi* strains, except for *T. evansi* type B, but it was believed that this *T. evansi* type B strain is not present in the study area and it was only observed in a specific place in Isiolo district in Kenya (Ngaira *et al.*, 2004).

PCR was performed under the following cycling conditions: an initial denaturation at 93°C for 3 min, followed by 30 cycles of 93°C for 30 sec (denaturation), 45°C for 30 sec (annealing), 72°C for 1 min. (extension) and 72°C for 5 min (final extension) (Chaudhry *et al.*, 2009).

PCR products were visualized by Agarose Gel Electrophoresis. A mixture of GelRed stain and 3 µL each of the PCR product were loaded in 2% agarose gel along with 100 bp DNA marker and was run at 100V for 30 minutes. The amplified products were visualized as a single compact band of expected size (~205) under UV light and documented by a gel documentation system installed in the FluorChem E

machine (ProteinSimple, Santa Clara, CA, United States).

cELISA test for the detection of *N. caninum* antibodies

N. caninum antibody in water buffalo sera were detected using a specific kit, competitive enzyme-linked immunosorbent assay (cELISA) (VMRD Inc., Pullman, WA, USA). Preparation of samples and test procedure were conducted following the manufacturer's instructions indicated on the test kit protocol.

This cELISA detects antibody to *N. caninum* in bovine sera. Sample serum antibody to *N. caninum* inhibits binding of horseradish peroxidase (HRP)-labeled *N. caninum* specific monoclonal antibody to *N. caninum* antigen coated on the plastic wells. Binding of the HRP-labeled monoclonal antibody conjugate is detected by the addition of enzyme substrate and quantified by subsequent color product development. Strong color development indicates little or no blockage of HRP-labeled monoclonal antibody binding and therefore the absence of *N. caninum* antibody in sample sera. Weak color development due to inhibition of the monoclonal antibody binding to the antigen on the solid phase indicates the presence of *N. caninum* antibodies in sample sera.

RESULTS AND DISCUSSION

Molecular detection of *T. evansi* using PCR

A total of 100 extracted DNA samples from water buffalo blood were tested for the presence of *T. evansi* using PCR and showed an overall positivity rate of 11% (11/100) in Nueva Ecija. Table 1 shows that, 4 out of 16 (25%) in Science City of Muñoz, 3 out of 13 (23.08%) in Sta. Rosa and 4 out of 16 (25%) in Talugtug tested positive while samples from Cabanatuan City, Carranglan, Lupao, San Jose City and

San Leonardo tested negative for *T. evansi* infection.

Table 1. PCR results for *T. evansi* detection

Collection Site	Total Number of Animals Tested	No. of Positive Animals	Percentage (%)
Cabanatuan City	7	0	0
Carranglan	5	0	0
Lupao	10	0	0
Science City of Muñoz	16	4	25
San Jose City	16	0	0
San Leonardo	17	0	0
Sta. Rosa	13	3	23.08
Talugtug	16	4	25
TOTAL	100	11	11

Figure 1 shows the gel electrophoresis result indicating positive cases of *T. evansi* in the tested DNA samples. Positive samples were revealed as bands lined in approximately 205 bp.

The PCR assay confirmed the parasite as *T. evansi*, the agent of Surra, as the amplicons displayed the specific ~205 bp band under UV transilluminator after gel electrophoresis.

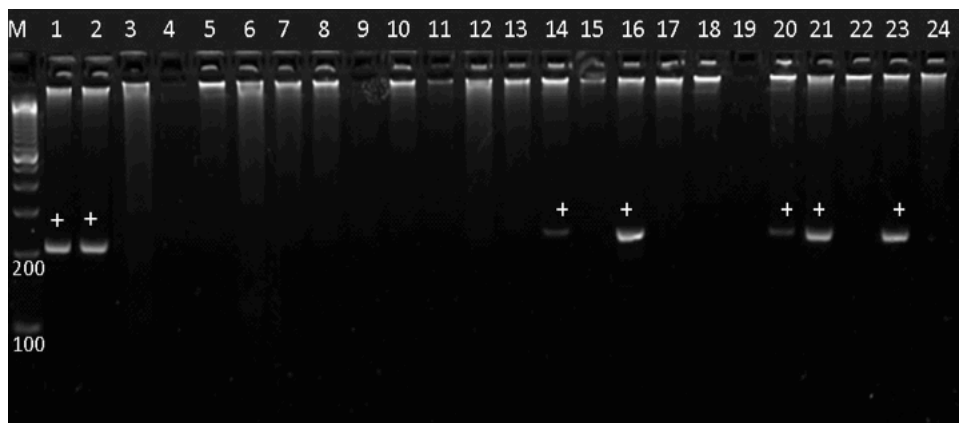


Figure 1. Agarose gel visualization of the PCR amplification (~205bp) for the detection of *T. evansi* *Rotat 1.2* VSG gene. Marker -100bp DNA ladder; lane 1 is the positive control while lanes 2, 14, 16, 20, 21 and 23 are positive samples at ~205 target bp.

Eight (8) water buffaloes in Talugtug and Science City of Muñoz were found to be positive with *T. evansi* using PCR. These two areas are geographically close to each other which may imply that the organism may have a higher chance of being transmitted into other water buffaloes within the area or the surrounding municipalities in the region. This imposes potential economic losses to the farmers because of decreased draught output, milk

production and reproduction losses of infected buffaloes as effect of having the organism (Dargantes et al., 2009). In the Philippines, tabanid fly or horsefly is the primary transmitter of *T. evansi* (Baticados et al., 2011a) but other blood-sucking insects like stable fly (*Stomoxys calcitrans*), buffalo fly (*Haematobia* spp. and mosquito (Manuel, 1998) are also believed to transmit the parasite mechanically. Tabanids are widely distributed in the

country and its population greatly increases during rainy season (OIE, 2009), coincidentally same season where the blood samples were collected for this study.

The result of this study supports some prevalence studies regarding Surra cases in Nueva Ecija. The present findings of this study showed remarkably higher positivity rate in Science City of Muñoz (25%), Sta. Rosa (23.08), and Talugtug (25%) compared to the prevalence study of Parayao (Unpublished) in Talavera (14.53%), and Miguel (Unpublished) in Rizal (16.84%). Meanwhile, Lagasca (Unpublished) reported a high prevalence of trypanosomosis in San Jose City (25.93 %) in which the current study detected none in the same city.

Moreover, in this study, the overall positivity rate of *T. evansi* infection in Nueva Ecija is 11% which is higher compared to the study of Baticados et al. (2011b) who reported a prevalence of 7.14% (3/42) of Surra among cattle in Saguday, Quirino using PCR and Medrano (Unpublished) who reported 7.33% and 10.19% *T. evansi* infection prevalence among cattle and swamp buffaloes, respectively, in Aurora province using microscopy. Konnai et al. (2008) conducted a survey of abortifacient infectious agents in livestock in Luzon, Philippines. From the study, among the 105 buffalo samples

collected, 1.9% (2/105) was tested positive with *T. evansi*. Likewise, based on the study conducted by Baticados et al. (2011a) on parasitological and PCR detection of *T. evansi* in water buffaloes from Luzon, Philippines, two of the 145 samples (0.13%) were positive for *T. evansi* in both, blood parasite examination and PCR.

The economic importance of *T. evansi* infection in the ruminant industry is most obvious when the disease already causes mortality, but even the sub-clinical infections have been shown to cause high losses from reduced feed efficiency and weight gain low milk production, and poor reproductive performance (Wint et al., 2008).

Detection of antibodies to N. caninum using cELISA

Results showed that 46 out of 100 serum samples (46%) from different cities and municipalities of Nueva Ecija were tested seropositive. Table 2 shows that, 4 out of 7 (57.14%) water buffaloes in Cabanatuan City, 22 out of 51 (43.14%) water buffaloes in Science City of Muñoz, 4 out of 10 (40%) water buffaloes in Sta. Rosa, 6 out of 12 (50%) water buffaloes in Sto. Domingo and 10 out of 20 (50%) water buffaloes in Talugtug recorded to have antibody titers against *N. caninum*.

Table 2. cELISA results for the detection of antibodies to *N. caninum* in serum sample of water buffaloes collected from 2017 to 2018.

Collection site	Total number of animals tested	No. of positive	Percentage (%)
Cabanatuan	7	4	57.14
Science City of Muñoz	51	22	43.14
Sta. Rosa	10	4	40
Sto. Domingo	12	6	50
Talugtug	20	10	50
TOTAL	100	46	46

Serum antibody from the sample inhibits the binding of enzyme-labeled

monoclonal antibody to *N. caninum* antigen coated on the plastic wells.

Figure 2 shows the computed percentage inhibition of antibodies, serum samples that reached the threshold of 30% inhibition (broken line) indicates a seropositive result.

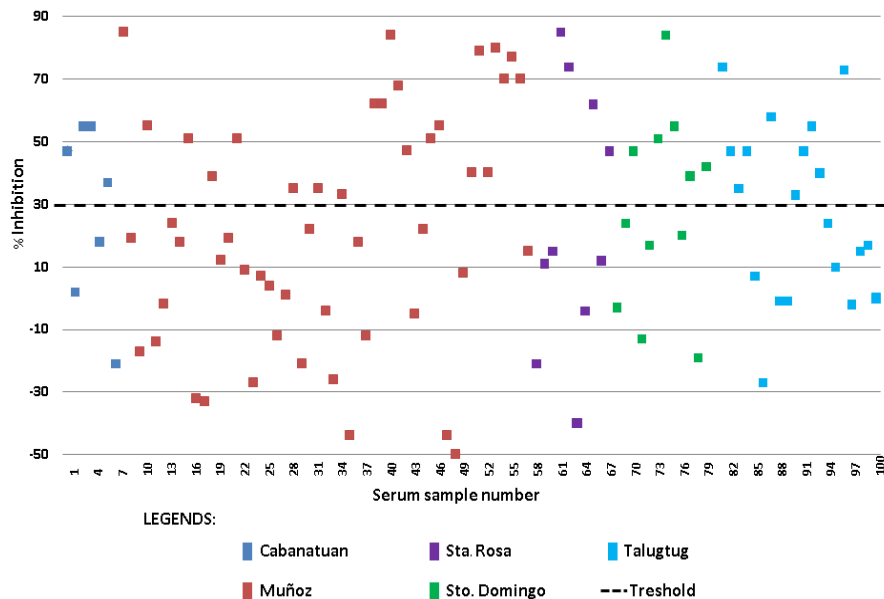


Figure 2. Percent inhibition values of *N. caninum* antibodies detected by cELISA in Water buffaloes from Nueva Ecija, Philippines collected from 2017-2018.

N. caninum is considered to be one of the major causes of abortion in cattle (Dubey et al., 1996) and implicated also in abortion cases in water buffalo (Guarino et al., 2000). In this study, a high proportion of seropositive animals from all the municipalities detected seropositive for *N. caninum* (Table 2), this is higher compared to the individual level seroprevalence study conducted by Abes and Divina (2008) in Nueva Ecija (27.3%) which included the municipalities of Aliaga, Cabanatuan City, Carranglan, Cuyapo, General Tinio, Guimba, Llanera, Science City of Muñoz, Quezon, Rizal, San Jose, Sto. Domingo, Talavera and Talugtug. Presence of *N. caninum* antibodies has been already investigated in other countries and included as a differential diagnosis for abortion cases among water buffaloes (da Silva, et al., 2017). This study showed that the total seropositivity rate of *N. caninum* antibodies in Nueva Ecija is 46%; this was higher compared to the prevalence

reported by Sadrebazzaz et al. (2004) in Mashhad, Iran with a prevalence of 15.18%. Nam et al. (2012) reported that 4.5% of swamp buffaloes tested was positive in northeastern Thailand. Sevgili and Gul Altas (2005) found antibodies to *N. caninum* in 23 of the 305 (7.5%) cow sera based on ELISA test results in the province of Sanliurfa, Turkey. According to the serological surveys, the prevalence of *N. caninum* infection in water buffaloes were reported to be at 64% in Southeastern region of Brazil (Fujii et al., 2001), 1.5% in South of Vietnam (Huong et al., 1998), 68% in Egypt (Dubey et al., 1998), 34.6% in Southern Italy (Guarino et al., 2000) and 37% in Iran (Hajkolaei et al., 2007).

The difference in results between this study and others conducted on the river buffaloes could be due to the different tests used, age groups, geography, season, management and/or breeds. Importation of water buffaloes was also a factor for the possibility that these animals might have been infected

before they arrived in the country and then served as a source of infection to the definitive and intermediate hosts of *N. caninum* (Abes and Divina, 2008). Domestic and stray dogs, definitive hosts, on farms have an easy access to placentas and the ingestion of infected placentas and fetal tissues can lead to the shedding of *N. caninum* oocysts (McAllister et al., 1998; Dijkstra et al., 2001).

Other studies on the detection of antibodies to *N. caninum* through ELISA requires or uses confirmatory tests to provide a stronger evidence of exposure to infection for the reason that, the animals who are seropositive to ELISA does not necessarily mean that they were infected but it may indicate the occurrence of the organism (Abes and Divina, 2008). In a study conducted by Meenakshi et al. (2007) in India, they detected the presence of *N. caninum* antibodies using cELISA among water buffaloes with a prevalence of 52.3% in adults and 45.4% in heifers and later used Indirect Fluorescent Antibody Test (IFAT) to verify the ELISA results.

In this study, although confirmatory tests like IFAT were not conducted, the high seropositivity of animals to ELISA supports the assumption that this protozoal agent might be present in the province. Its economic importance in water buffalo industry, and detection of this parasite to dogs cohabitating with water buffaloes should also be investigated. Dogs are generally known as the definitive host of this parasite. They shed parasite oocysts in their feces which can be ingested by grazing water buffaloes through contaminated grass. The other way around, uninfected dogs can horizontally acquire *N. caninum* through ingestion of aborted materials like fetuses and placenta infected with the parasite. Farmers should be well aware of these modes of spread to create

measures that prevent disease occurrence.

CONCLUSION

In conclusion, the current study was able to provide a molecular and serological evidence of the presence of *T. evansi* and *N. caninum* antibodies, respectively, among water buffaloes in Nueva Ecija. However, further studies should be employed to determine whether the presence of *T. evansi* and antibodies to *N. caninum* in the province are associated to abortion. It is recommended that the quality of DNA-extracted samples for PCR should be checked targeting β -actin as housekeeping gene. DNA-extracted samples should also be identified using a more sensitive technique like DNA sequencing for confirmation of the parasite. Similar studies should consider expanding the number of samples to be collected and larger collection area.

Future studies should consider risk factor analysis, such as mode of transmission of this disease, potential vectors and intermediate hosts in the area, entry of new stock in the area through importation, animal movement and dispersal project practices, knowledge of the farmer to the disease, and grazing distance of one animal to another.

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INFORMATION NOTES

The experiment was approved by the Committee of Ethics on Animal Use of the sector of Agrarian Sciences of the Federal University of Paraná, Curitiba, PR, Brazil, under protocol n. 027/2017.

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