Neospora caninum and bovine neosporosis: current situation in vaccine research

Virginia Marugan-Hernandez

The Royal Veterinary College, University of London, Hawkshead Lane, North Mymms, AL9 7TA, UK

Summary

Neospora caninum, a tissue-cyst-forming parasite, is the causative agent of bovine neosporosis. It is considered to be one of the most important transmissible causes of reproductive failure in cattle; abortion and neonatal mortality result in significant economic losses within the cattle industry worldwide. The balance between acute (mediated by the tachyzoite stage) and persistent (mediated by the bradyzoite stage) phases of the infection is influenced by the immune-status of the animal, and for pregnant cows (the intermediate host) is critical for transplacental (vertical) transmission of the parasite and associated disease outcomes. The horizontal route from the definitive host, the dog, occurs via oocyst ingestion (with sporozoites within) and plays a minor but important role in transmission of the infection in cattle. Despite the importance of this disease, there is no vaccine or treatment currently available, and the only available control measure to reduce the impact of disease is informed management on the farm. The development of vaccines, targeting key biological processes such as invasion and persistent infection, is an urgently needed aid in the control of this widespread parasite.

Keywords: *Neospora caninum*, bovine neosporosis, transplacental transmission, vaccine candidates

Introduction

Neospora caninum is a protozoan parasite which belongs to the Apicomplexa *phylum* (Fig.1) and is the aetiological agent of bovine neosporosis. This disease causes reproductive failure in cattle and is considered to be one of the main causes of infectious abortion with a consequent significant economic impact on both the dairy industry and the meat sector.

Life cycle

Neospora caninum has a facultative heteroxenous life cycle involving a definitive (some canids e.g. dog, coyote and dingo) and certain intermediate hosts (e.g. cattle, sheep, horse, pig, and deer) for the development of sexual (definitive hosts) and asexual (definitive and intermediate hosts) stages of the parasite.

Tachyzoite, bradyzoite and sporozite stages (Table 1), contain the basic structures and organelles of eukaryotic cells; in addition they have components which are specific for the Apicomplexa *phylum*: conoid, inner membrane complex, apicoplast, micronemes, rhoptries and dense granules (Fig.2). The fast replicating tachyzoites disseminate within the host and are responsible for the acute phase of the infection. The bradyzoites, residing within tissue cysts replicate slowly and are responsible for the persistence of the parasite in cattle. The sporozoites are contained within the oocyst which is the structure responsible for parasite survival in the environment and for horizontal transmission between definitive host and intermediate hosts. Kul and colleagues have recently described enteroepithelial stages that give rise to sporozoites in the definitive host (schizogony, gametogeny and zygote formation) in naturally infected dogs.

The definitive host sheds unsporulated oocysts that then undergo sporogony to become infective (Fig.3). When ingested by an intermediate host the sporozoites will be released and transform into tachyzoites which are able to proliferate and disseminate to different organs. This phase will end once the host develops a protective immune-response, causing transformation of tachyzoites into the more

slowly proliferating bradyzoites which develop within the tissue cyst, generally confined to nervous tissue and skeletal muscle, to establish the persistent phase of infection.

Transmission

Transmission in cattle can occur in two ways: horizontally by the ingestion of oocysts or vertically via the placenta (transplacental transmission) in pregnant cows (Fig.4). Transplacental transmission can be classified depending on the origin of the infection as endogenous (EnTT) or exogenous (ExTT). EnTT is caused by the reactivation of tissue cysts in a previously (persistently) infected animal and is associated with an endemic pattern of reproductive failure and maintenance of infection within the herd. In contrast, ExTT occurs following a primary horizontal infection (oocysts are ingested by a pregnant animal) and is associated with an epidemic pattern of abortion. Transmission to the definitive host mainly occurs following ingestion of foetal or placental tissues infected with tissue cysts. Infections by *N. caninum* have also been detected in wild animals, evidencing the ability of the parasite to circulate between domestic and wild animals.

Pathogenesis

A natural infection with *N. caninum* is asymptomatic in adult animals and the only clinical sign is abortion in pregnant cows and neurological signs in infected calves. The main factors influencing the pathogenesis of the disease are related to parasite invasion, replication and establishment within the host cell (intraspecific variation between isolates has shown different presentations and severity of the disease) and the ability of the host to mount an efficient immune-response to inhibit parasite proliferation and persistence.

As a consequence of *Neospora* infection the host develops a complex immune-response against the the parasite. The intracellular location of *N. caninum* promotes a cell-mediated immune-response (Th1) critical for protection against the disease and characterised by the production of pro-inflammatory cytokines (e.g. IFN-γ). At mid-gestation a down-regulation of cell mediated immunity occurs to avoid foetal rejection. This situation causes a variation in Th1/Th2 balance which can permit a reactivation of

quiescent bradyzoites to differentiate into tachyzoites and allow dissemination of the parasite to the placenta and the foetus. This Th1/Th2 imbalance also makes pregnant cows more susceptible than non-pregnant animals to a primary infection (horizontal) with *Neospora.*

With neosporosis the clinical outcome is related to the period of the pregnancy in which the primary infection or reactivation occurs. Foetal immune-competency is not apparent until the first third of the pregnancy is completed; as a consequence, the foetus is especially vulnerable in this initial period, and death and reabsorption are frequent in cases of infection by *N. caninum*. The highest rate of abortion and transplacental transmission leading to congenitally infected calves with clinical signs occurs in the second third of pregnancy, when the foetal immune system is still inchoate. If the infection takes place in the last third, the foetus is capable of survival, however, transplacental transmission is highly probable leading to healthy calves that are congenitally infected, and may contribute to the prevalence of *Neospora* if retained as breeding stock on the farm.

Pathological findings and clinical signs

Abortion can occur as a consequence of different factors affecting the foetus and/or the placenta: a) parasite proliferation damaging essential foetal tissues or the placenta, b) insufficient oxygenation or nutrition of the foetus due to placental damage, c) release of maternal prostaglandins causing luteolysis and therefore abortion, d) alteration of placental immunity associated with the release of maternal pro-inflammatory cytokines which can cause rejection of the foetus.

Foetuses lost in early pregnancy are frequently autolysed and the infected placenta often presents with multifocal necrotic foci within the cotyledons combined with intact intercotyledonary areas. The necrotic foci normally show areas of intense inflammatory infiltration by mononuclear cells and dystrophic calcification. These cellular infiltrations begin in the maternal caruncles and then extend to the foetal cotyledon, with the appearance of areas of haemorrhage and necrosis. In aborted foetuses and stillbirths most commonly lesions are found in the brain (Fig.5) and consist of perivascular cuffs, gliosis and distinct multifocal small areas of liquefactive necrosis surrounded by gliosis. Less frequently,

similar non-suppurative foci of inflammation can also be seen in the heart (Fig.5), liver or rarely in the kidney, skeletal muscle or lung. Congenitally infected calves with clinical signs more frequently have inflammatory lesions in the spinal cord and less frequently in the brain; hydrocephalus may also be present, explaining the ataxia, decreased reflexes and paralysis that may be observed. In calves without clinical signs and adult animals, lesions are largely confined to tissue cysts found mainly in the brain and less frequency in the heart, liver and muscles.

Control and vaccination

In the absence of an effective treatment or vaccine against bovine neosporosis the control of the disease relies on management measures on the farm. Control programmes should be adapted to the specific situation, for which serological diagnosis is essential for identifying the extent of infection in the herd and whether EnTT and/or ExTT is occurring. The main control measures to prevent EnTT must focus on avoiding the reactivation of bradyzoites in persistently infected animals by maintaining optimal biosecurity and good management to avoid stresses that may favour immunosuppression. Selection of seronegative animals for the replacement of breeding stock is another important control measure. The risk of ExTT may be reduced by controlling access of dogs and other potential definitive hosts to the farm along with safe disposal of contaminated material (e.g. foetal or placental tissues) and the control of other intermediate hosts (e.g. rodents).

Various drugs and natural extracts have been tested *in vitro* or in different animal models but their efficacy has not been proven in natural infections in cattle. Recently antimalarial drugs and bumped-kinase inhibitors have been shown to reduce both proliferation *in vitro* and transplacental transmission in pregnant mouse models.

Currently, the best control option is to develop a vaccine designed to prevent both abortion and transplacental transmission. Inactivated or classic subunit vaccines against neosporosis are safe but currently do not stimulate a protective immunity; the only commercialised vaccine (Neoguard®), based on inactivated tachyzoites, has been withdrawn from the market due to low efficacy and ambiguous

results. As with ovine toxoplasmosis, for which a live vaccine (based on tachyzoites of the S48 incomplete strain) is commercially available, live vaccination has delivered the best immune-protection to date. In order to avoid safety problems (such as reverting to virulence during pregnancy) low virulence isolates with a reduced capacity to persist in the host have been proposed as potential candidates; this attenuation can be natural (isolates from asymptomatic animals) or induced by subsequent manipulation (temperature sensitive strains, multiple passage in cell culture, gamma irradiation or transgenic manipulation). However, so far none have been licensed.

In this context, new generation vaccines (recombinant proteins, DNA or vectored), safe and easy to manufacture, are preferred as the principal line of research in vaccine development. The main concern for their development is the pre-selection of candidates that can confer immune-protection. Recently, *in silico* tools are becoming available for the prediction of vaccine candidates, but their successful application has not been demonstrated yet. Thus lacking certain candidate predictions, proteins involved in the lytic cycle of the tachyzoite (Fig.6) and potentially involved in host cell adhesion/invasion and parasitophorous vacuole formation (surface proteins and/or those secreted from micronemes, rhoptries or dense granules; Fig.2) have been the most studied for their protective potential. These experimental vaccines are designed to block specific processes by triggering an immune-response targeting specific proteins involved in vital mechanisms and so preventing parasite proliferation. So far several different proteins have been tested showing variable results depending on the expression system, route of administration and/or adjuvants used. Hemphill and colleagues have reviewed the different candidates tested over the last twenty years. To a lesser extent, proteins of the bradyzoite stage have also been studied and tested as vaccines with the aim of targeting persistent infections but with little success to date.

In this scenario of partial success, new approaches are being integrated as are the combination of different antigens, use of improved adjuvants and enhancers of the immune response (e.g. fusion to TLR-ligands) or combinations of live parasites with new drugs. It is also important to highlight the

increasing interest for DIVA vaccines (differentiating infected from vaccinated animals) which should be taken into account in vaccine development against bovine neosporosis.

Conclusions

Transplacental transmission is the principal mechanism in the etiology of bovine neosporosis. Endogenous transmission (EnTT) is more likely to occur in cattle that were congenitally infected in comparison to those which were infected postnatally (when non-pregnant). This could be due to the development of an incomplete immunity following a foetal infection; therefore a better understanding of the development of immune-protection in the host at different stages is essential. Curiously, this situation is significantly different to that in *Toxoplasma gondii*, for which EnTT seems to play a minor role. Targeting and eliminating the persistent phase of infection at first site would seem to be a useful objective in *Neospora* research. However, most of the chemicals and vaccine candidates tested target the acute phase of the infection (tachyzoites) rather than the bradyzoites protected within the tissue cyst. Advances in the diagnostic tools for *N. caninum* have provided a good idea of the global impact of neosporosis and helped significantly with its control. Unfortunately this is not always enough and vaccination still appears to be the best option for reducing losses. The development of new generation vaccines is currently constrained by partially successful trials and a lack of novel candidates in the pipeline. Many of the mechanisms involved in the *Neospora*-host interaction have been inferred from *T. gondii*, but it is now clear that there are significant differences between these two parasites and the scientific community needs to further explore the biology of *Neospora* to identify new potential vaccine immunogens. Recent proteomic and transcriptome data and further advances in genetic modification technologies should also help the identification these candidates along with novel animal models (e.g. other ruminants) and so increase the scope for evaluating potential new formulations.

Further Reading

Darwich, L., Li, Y., Serrano-Perez, B., Mur-Novales, R., Garcia-Ispierto, I., Cabezon, O., Lopez-Gatius, F. and Almeria, S. (2016). Maternal and foetal cytokine production in dams naturally

and experimentally infected with *Neospora caninum* on gestation day 110. *Res Vet Sci,* **107,** 55-61.

Donahoe, S. L., Lindsay, S. A., Krockenberger, M., Phalen, D. and Slapeta, J. (2015). A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. *Int J Parasitol Parasites Wildl,* **4,** 216-238.

Dubey, J. P., Buxton, D. and Wouda, W. (2006). Pathogenesis of bovine neosporosis. *J Comp Pathol,* **134,** 267-289.

Goodswen SJ, Barratt JL, Kennedy PJ, Ellis JT: Improving the gene structure annotation of the apicomplexan parasite Neospora caninum fulfils a vital requirement towards an in silico-derived vaccine. *International journal for parasitology* 2015, **45**(5):305-318.

Hemphill A, Aguado-Martinez A, Muller J: Approaches for the vaccination and treatment of Neospora caninum infections in mice and ruminant models. *Parasitology* 2016, **143**(3):245-259.

Kul, O., Atmaca, H. T., Anteplioglu, T., Ocal, N. and Canpolat, S. (2015). *Neospora caninum*: the First Demonstration of the Enteroepithelial Stages in the Intestines of a Naturally Infected Dog. *J Comp Pathol,* **153,** 9-13.

Reichel, M. P. and Ellis, J. T. (2009). *Neospora caninum*--how close are we to development of an efficacious vaccine that prevents abortion in cattle? *Int J Parasitol,* **39,** 1173-1187.

Thilsted, J. P. and Dubey, J. P. (1989). Neosporosis-like abortions in a herd of dairy cattle. *J Vet Diagn Invest,* **1,** 205-209.

Trees, A. J. and Williams, D. J. (2005). Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. *Trends Parasitol,* **21,** 558-561.

Winzer, P., Muller, J., Aguado-Martinez, A., Rahman, M., Balmer, V., Manser, V., Ortega-Mora, L. M., Ojo, K. K., Fan, E., Maly, D. J., Van Voorhis, W. C. and Hemphill, A. (2015). In Vitro and

In Vivo Effects of the Bumped Kinase Inhibitor 1294 in the Related Cyst-Forming Apicomplexans *Toxoplasma gondii* and *Neospora caninum*. *Antimicrob Agents Chemother,* **59,** 6361-6374

Acknowledgments

The author thanks Laura Polledo and Jorge Gonzalez (Micros Veterinaria) for the micrographs of the paraffin sections and David Buxton for his suggestions in the manuscript.

Conflict of interests

The author declares that she has no competing interests.

Table 1. Principal characteristics of the invasive stages of *Neospora caninum*

Characteristics	Invasive stage		
	Tachyzoite	Bradyzoite	Sporozoite
Containing structure	parasitophorous vacuole	tissue cyst	oocyst
Localization in host	different cell types	nervous tissue and skeletal muscle tissue	intestine
Structures size / wall thickness (μm)	variable	107 / 4	10-12 / 0.6-0.8
No. of zoites / structure	2-100	up to 200	8
Zoite length (μm)	5-7	6-8	6-7
Zoite width (μm)	1-5	1-2	2
No. of micronemes	~ 150	~ 150	indeterminate
No. of rhoptries	8-18	> 8-18	indeterminate
No. of dense granules	~ 15	8-10	~ 15
Nucleus position	central	sub-terminal	central or slightly posterior
Cellular division	endodyogeny	asinchronic endodyogeny	schizogony

Figure legends

Figure 1. Taxonomic classification of *Neospora caninum* and main characteristics of each organizational level

Figure 2. Principal structures and organelles of *Neospora caninum*

Figure 3. Life cycle and stages of *Neospora caninum*. Sporulated oocysts previously shed by the definitive host (dog) are ingested by an intermediate host (cow) where sporozoites differentiate into tachyzoites which disseminate to different organs (acute phase of infection). Host immune-response causes the transformation of tachyzoites into bradyzoites within tissue cysts (persistent phase of infection) which can in turn infect the definitive host following their ingestion.

Figure 4. Transmission of *Neospora caninum*. Vertical transmission from the cow to offspring can occur by endogenous (EnTT, from persistently infected animals) or exogenous (ExTT, from primary infected animals) transplacental transmission. The infection can cause the death of the foetus or calf which can in turn potentially infect the definitive host. Congenitally infected calves without clinical signs can contribute to the maintenance of infection within the herd as well as having the potential to transmit horizontally to other domestic or wild species.

Figure 5. Light micrographs of histological sections stained with haematoxylin and eosin. A. Brain, at the level of the thalamus showing non-suppurative encephalitis with a small focus of necrosis surrounded by glial cells. B. Higher magnification of the necrotic focus in A. C. Heart, left myocardium, showing a multifocal non-suppurative interstitial inflammatory infiltrate. D. Higher magnification of the inflammatory infiltrate in C. The bar indicates the magnification of each image.

Figure 6. Lytic cycle of *Neospora caninum*. Tachyzoites interact with the host cell by gliding motility mediated by the cytoskeleton to establish an initial contact using the surface proteins and those secreted by the micronemes. After reorientation the invasion process starts with the involvement of proteins of the micronemes and the neck of the rhoptries. At this point the tachyzoite secretes proteins from the bulb of the rhoptries and the dense granules into the nascent parasitophorous vacuole for its formation and communication with the host cell. Once intracellular the tachyzoite will divide by endodyogeny to

eventually cause the lysis of the host cell releasing new tachyzoites and either continuing with the lytic cycle; or developing into tissue cysts with bradyzoites (persistent phase) as consequence of an efficient immune response.

Figure 1.

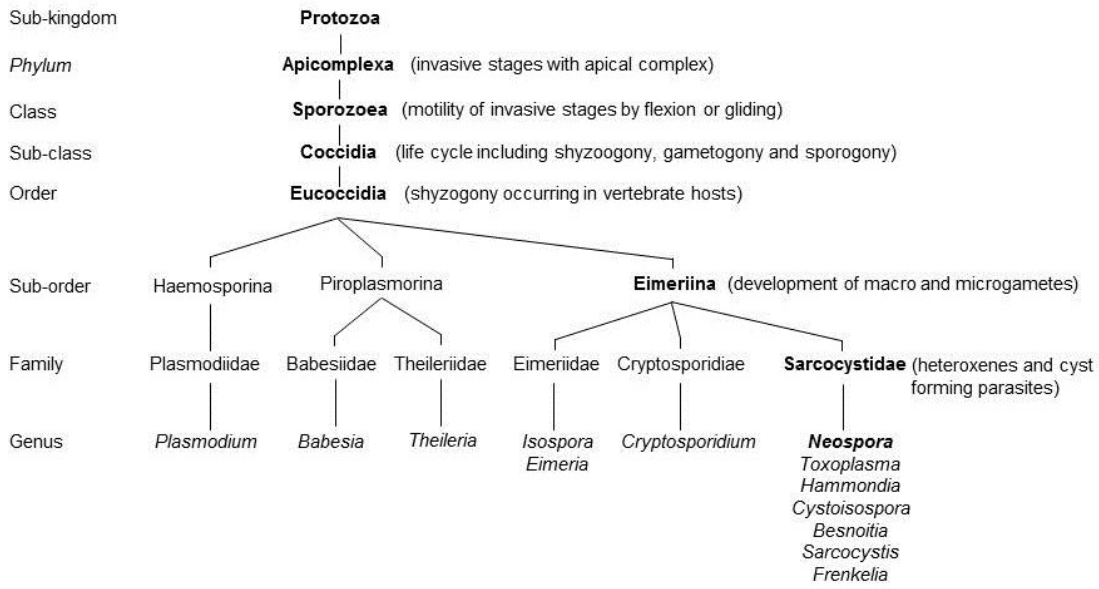

Figure 2.

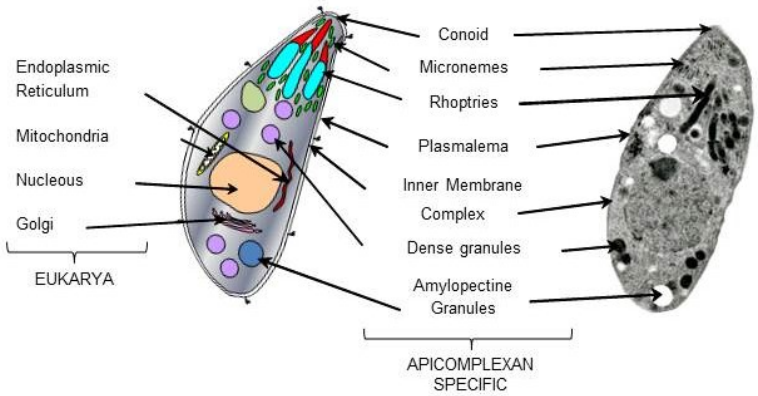

Figure 3.

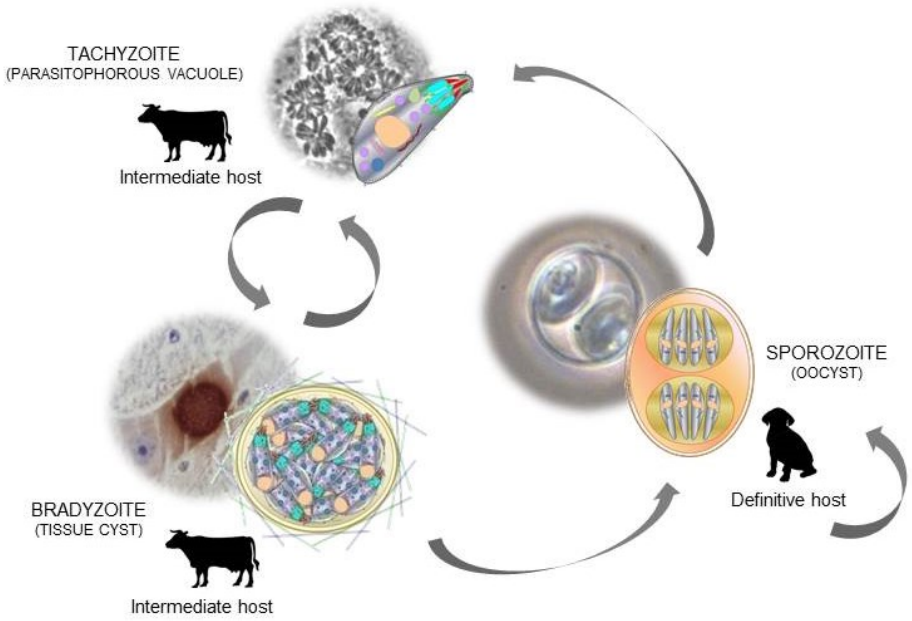

Figure 4.

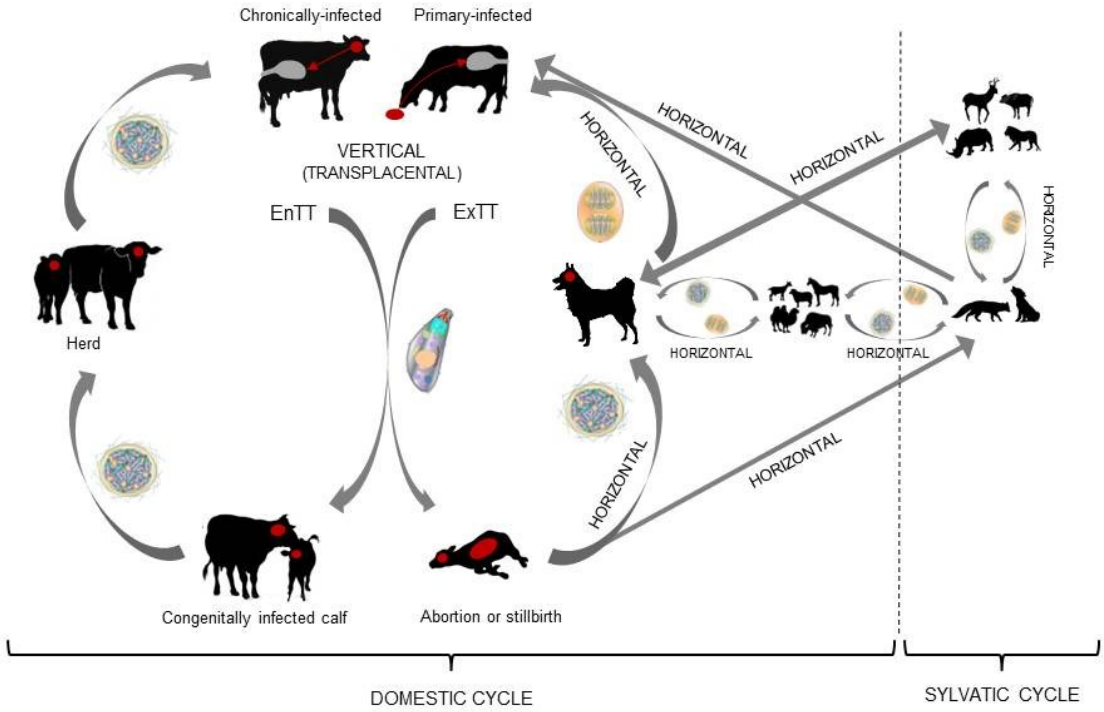

Figure 5.

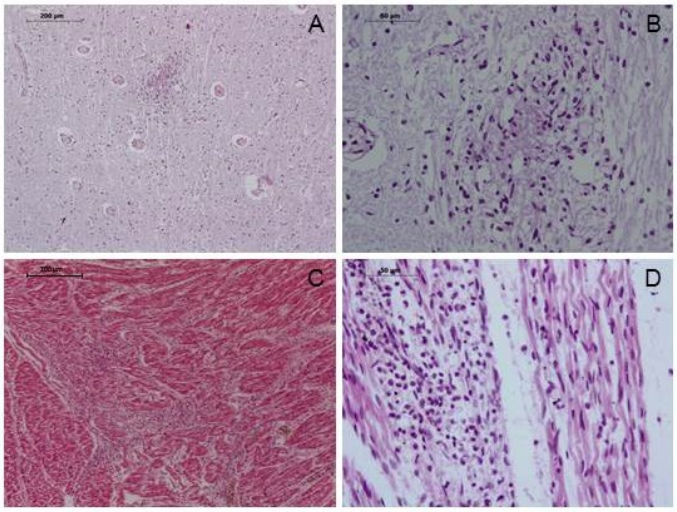

Figure 6.