

Seroprevalence of *Toxoplasma gondii* and *Neospora* spp. in equids from three municipalities in Pará, Brazil

Emma Norlander



*Uppsala
2014*

Degree Project 30 credits within the Veterinary Medicine Programme

*ISSN 1652-8697
Examensarbete 2014:72*

Seroprevalence of *Toxoplasma gondii* and *Neospora* spp. in equids from three municipalities in Pará, Brazil

Seroprevalens av *Toxoplasma gondii* och *Neospora* spp. hos hästdjur från tre kommuner i Pará, Brasilien

Emma Norlander

Supervisor: Ulf Emanuelson, Department of Clinical Sciences

Examiner: John Pringle, Department of Clinical Sciences

Degree Project in Veterinary Medicine

Credits: 30 hec

Level: Second cycle, A2E

Course code: EX0736

Place of publication: Uppsala

Year of publication: 2014

Title page photo source: Emma Norlander

Number of part of series: Examensarbete 2014:72

ISSN: 1652-8697

Online publication: <http://stud.epsilon.slu.se>

Key words: *Toxoplasma gondii*, *Neospora*, horse, donkey, mule, Brazil, Pará

Nyckelord: *Toxoplasma gondii*, *Neospora*, häst, åsna, mula, mulåsna, Brasilien, Pará

**Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences**

Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences

ABSTRACT

This study presents a survey of the seroprevalence of *Toxoplasma gondii* and *Neospora* spp. in equids in three municipalities in Pará, a Brazilian state with the Amazonian rainforest and the Amazon River as dominating features. *Toxoplasma gondii* and *Neospora* spp. are two closely related parasitic protozoans not separated as different genera until 1988. *Neospora* spp. includes two species, *N. caninum* and *N. hughesi*, that are impossible to distinguish between based solely on serology. Seroprevalence in previous studies from other areas of Brazil has presented a prevalence ranging from 5.9% to 43.2% for *T. gondii* and 0% to 15.9% for *Neospora* spp.

Blood samples from a total of 440 horses, mules and donkeys from three different categories were sampled; urban horses, farm horses and sport horses. The samples were screened for antibodies using indirect fluorescent antibody test (IFAT), using a cut-off value of 1:16 for *T. gondii* and 1:50 for *Neospora* spp. The samples were tested in two-fold titrations until reaching end-point titer.

Overall prevalence for *T. gondii* in the present study was 6.4% (28/440) and for *Neospora* spp. 4.5% (20/440), results that are among the lowest compared to prevalence studies made in other areas of Brazil. The end-point titers for *T. gondii* were in the lower end of the spectra, with 32.1% (9/28) of the positive samples having an end-point titer of 1:16 and the highest end-point titer being 1:1024. Analysis for *Neospora* spp. resulted in high end-point titers, only 5.0% (1/20) of the seropositive individuals having an end-point titer of 1:50 and the highest titer being 1:2800. The prevalence on the basis of different determinants gave statistically significant differences in frequencies in several determinant groups. For *T. gondii*, the prevalence in sport horses was higher than in urban horses. The prevalence of *Neospora* spp. was higher in males than females, in urban horses compared to farm horses and the prevalence in the municipalities of both Santarém and Óbidos was significantly higher than in Brasil Novo. Additional research is necessary to determine whether these statistically significant differences are reflecting reality or if they are a consequence of the study design. More studies are also needed to be able to estimate the true prevalence in Pará and in Brazil in general. This knowledge is of importance when calculating the risk of meat from infected horses causing clinical disease in humans.

SAMMANFATTNING

Detta är en seroprevalensstudie avseende *Toxoplasma gondii* och *Neospora* spp. hos hästdjur från tre kommuner i Pará, en brasiliansk stat som karaktäriseras av regnskog och amazonfloden. *Toxoplasma gondii* och *Neospora* spp. är två närbesläktade protozoer som inte separerades som genus förrän 1988. Genus *Neospora* utgörs av två arter, *N. caninum* och *N. hughesi*, som inte kan särskiljas endast genom serologi. Tidigare studier gjorda i andra områden av Brasilien har presenterat prevalenser på 5.9% till 43.2% för *T. gondii* och 0% till 15.6% för *Neospora* spp.

Blodprover samlades in från totalt 440 hästar, åsnor, mulåsnor och mulor i tre olika kategorier; stadshästar, hästar från gårdar och sporthästar. Proverna analyserades för antikroppar genom indirect fluorescent antibody test (IFAT). Cut-off värdet sattes till 1:16 för *T. gondii* och 1:50 för *Neospora* spp. Proverna testades i dubbla titreringar tills det att maximal titer uppnåts.

Den totala prevalensen för *T. gondii* i denna studie var 6.4% (28/440) och 4.5% (20/440) för *Neospora* spp. Detta är en relativt låg frekvens i jämförelse med studier som gjorts på hästar i andra områden i Brasilien. Maximala titrarna för *T. gondii* var liknande de som setts i andra studier, 32.1% (9/28) av de positiva proverna hade en maximal titer på 1:16 och den högsta titern var 1:1024. Analyseringen av proverna för *Neospora* spp. visade höga maximala titrar jämfört med andra studier, endast 5.0% (1/20) hade maximal titer på 1:50 och den högsta maximala titern var 1:2800. Prevalensen baserad på olika förutbestämda faktorer visade på en statistiskt signifikant skillnad inom flera grupper. För *T. gondii* var prevalensen signifikant högre hos sporthästar jämfört med stadshästar. Gällande *Neospora* spp. var prevalensen högre hos handjur jämfört med hondjur samt hos stadshästar jämfört med hästar från gårdar. Dessutom var prevalensen i båda kommunerna Santarém och Óbidos högre än i Brasil Novo. Ytterligare studier behövs för att kunna avgöra om detta är verkliga skillnader eller om de är ett resultat av studiens utformning. Det behövs också fler studier för att kunna uppskatta den sanna prevalensen i Pará och Brasilien. Kunskap om prevalens behövs bland annat för att kunna avgöra om intag av hästkött utgör en risk för klinisk sjukdom hos människor.

Innehållsförteckning

Introduction	1
Objective	1
Literature review.....	2
Toxoplasma gondii.....	2
Hosts.....	2
Stages of the parasite.....	2
Life cycle.....	2
Transmission	4
Consequences of infection.....	4
Diagnosis.....	5
Neospora.....	5
Hosts.....	5
Transmission and life cycle.....	6
Consequences of infection.....	6
Diagnosis.....	7
Seroprevalence of Toxoplasma gondii and Neospora spp. in horses in Brazil	8
Horse categories in the state of Pará	10
Material and methods.....	12
Site of sample collection	12
Study design	13
Population definition	13
Sampling method.....	13
Sample size.....	13
Sample collection	14
Laboratory technique for antibody detection	15
Cultivation of tachyzoites and preparation of slides	15
Toxoplasma serology	15
Neospora serology.....	15
Visualization and interpretation	16
Statistical analysis	16
Results	17
Toxoplasma gondii prevalence and titers.....	17
Neospora prevalence and titers.....	17
Prevalence on the basis of determinants.....	18
Discussion.....	21
Seroprevalence	21
End-point titers.....	22
Seroprevalence on the basis of determinants.....	23
Laboratory analysis	25

Additional data	25
Consequences of neosporosis and toxoplasmosis in horses	25
Conclusions.....	25
Acknowledgements	27
References.....	28
Oral sources.....	31

INTRODUCTION

Today, Brazil is not only the sixth largest and one of the fastest growing economies in the world but is also working hard at reducing social and economic inequality (UNESCO, 2011; Fagge, 2011). Nevertheless, Brazil is still a developing country. The most visible poverty is represented by favelas and slums in the urban areas, where an estimated 5% of the population is classified as extremely poor. This has to be compared to an estimate of 25% in rural communities, which are mainly located in the least developed areas in the northeast of Brazil and isolated parts of Amazonas. Of these poor rural households, smallholder farms make up nearly 40% and they rely on their farms for surviving (IFAD, 2011). A small number of large landowners monopolize most rural areas in the country but they coexist with millions of small landowners, landless workers and rural workers living in unsafe conditions (Beghin, 2008).

The state of Pará is situated in the amazon region in the north of Brazil. The dominating feature of the Pará area is the waste rainforest and the Amazon River, passing through the state from west to east before entering the Atlantic Ocean. The population is concentrated to the few cities and towns. In addition to these there are a few settlements, trading posts, plantations and small groups of Indians in the state (Encyclopaedia Britannica, 2012). The state of Pará is the most important cattle-producing region in Northern Brazil and accounts for the fifth largest herd in Brazil, with 17 million animals. The cattle production is the most important economic activity in more than 50% of the municipalities in Pará, with approximately 1.1 million cattle slaughtered per year (Hamad Minervino, 2012, personal communication).

The population of equidae in the state of Pará is estimated to more than 280 000 animals, making these animals play a major socio-economic roll in this area of Brazil. Since many people in the state are struggling economically, the horses are often of great importance in daily life and sudden poor health can further reduce a potentially already low income. Among the diseases that affect horses, parasitic and infectious diseases stand out as major causes of damage (Hamad Minervino, 2012, personal communication).

OBJECTIVE

The aim of this study was to survey the seroprevalence of *Toxoplasma gondii* and *Neospora* spp in horses in three municipalities in the state of Pará, Brazil. The information gained was also to be used to analyze whether there were any difference in seroprevalence between cities, between different categories of horses as well as difference in seroprevalence between sex, age and breeds of horses.

This study was performed as a part of a more comprehensive study including the 26 largest municipalities in the state of Pará, Brazil. In addition to the pathogens included in this study, the aim of larger study was to survey several other pathogens such as *Rickettsia* spp. In charge of the larger study was prof. Antonio Humberto Hamad Minervino, Federal University of Western Pará (UFOPA).

LITERATURE REVIEW

Toxoplasma gondii and *Neospora* spp. are two closely related parasitic protozoans of the phylum Apicomplexa. Protozoans are unicellular organisms and those belonging to the phylum Apicomplexa are characterized partially by their intracellular occurrence (Taylor et al., 2007).

Toxoplasma gondii

Toxoplasma gondii is a parasite prevalent in most areas of the world, causing infections in both humans and animals. The parasite's zoonotic qualities and its lack of species-specificity make it a highly important infectious agent in both veterinary and human medicine. *T. gondii* is the only species in genus *Toxoplasma* (Dubey, 2009).

Hosts

The definitive host for *T. gondii* is felines, both domestic cats and most other species of felids. Virtually any other warm-blooded animals, such as other mammals (including humans) and birds, can get infected but acts only as intermediate hosts and will not pass oocysts in their feces. Instead, these intermediate hosts may develop infective tissue cysts that persists for life and may cause disease. More importantly, *T. gondii* has the ability to cause abortions and foetal infections in humans (Dubey, 2009).

Stages of the parasite

The different stages of *T. gondii* are primarily tachyzoites, bradyzoites and sporozoites (in oocysts). There are several other stages, but these are the three only stages capable of infecting a host (Dubey et al., 1998). Tachyzoites are the stage where the parasite reproduces asexually at a fast rate and is mostly prevalent during the acute phase of infection. The tachyzoites are crescent-shaped when viewed in a microscope, about 2-6 µm in length and contains different organelles, inclusion bodies and a nucleus. Bradyzoites are smaller than tachyzoites and are mostly found during the chronic phase, but are structurally not very different from tachyzoites. In this stage the parasite is encysted in tissue, where it multiplies slowly. The so-called tissue cysts reside intracellularly and each contains as little as two or as many as thousands of bradyzoites, depending on the age of the cyst (Dubey, 2009). Oocysts occur in two different forms, non-sporulated and sporulated. As the nucleus in the non-sporulated oocyst divides, the oocyst becomes sporulated. When fully matured, the sporulated oocyst contains two sporocysts, each holding four sporozoites. Sporozoites are, like bradyzoites, structurally similar to tachyzoites (Dubey et al., 1998).

Life cycle

Together, the three stages of *T. gondii* form a complex life cycle, using a prey-predator system where the sexual reproduction occur in the definitive host, while the asexual stages is seen in the intermediate host (Afonso et al., 2006).

Life cycle in cats

Cats become infected by *T. gondii* through ingestion of tissue cysts (containing bradyzoites), tachyzoites or oocysts. However, the likelihood of a cat shedding oocysts after ingestion of *T.*

gondii varies strongly depending on which stage of the parasite was ingested. Bradyzoites are the most infective stage to cats with almost all cats shedding oocysts after ingestion, irrespective of dose. Ingestion of oocysts or tachyzoites leads to infection in less than 50% of the cases. This is explained by *T. gondii*'s adaption to transmission by carnivorousness, i.e. cats getting infected by eating infected prey (intermediate hosts) (Dubey, 2009).

Most infections occur post-natally through ingestion as discussed above, but it is possible for cats to be congenitally infected (Dubey et al., 1996). Studies have also shown that experimentally infected cats can pass tachyzoites in the milk, suggesting a possibility of suckling kittens becoming infected after birth (Powell et al., 2001).

The cycle in cats depends upon which stage of *T. gondii* was ingested, but the outcome for all successfully infected cats are shedding of oocysts. Only the bradyzoite-induced cycle has been completely researched and is the one discussed below. The cycle starts with ingestion of infected prey containing tissue cysts. After reaching the stomach and intestines the cyst wall is dissolved, making the bradyzoites free to penetrate small intestinal enterocytes. Inside the cells the development of several asexual stages of *T. gondii*, designated types A to E, precedes the initiation of gametogony and the sexual reproduction. Fertilization and forming of the oocyst wall occur prior to the bursting of the infected enterocyte, placing the non-sporulated oocyst within the intestinal lumen where it is to be excreted in the faeces. The oocyst sporulates outside the cat (Dubey, 2009). The oocyst wall is very well adapted to protect the oocyst from damage, which makes the parasite extremely resilient in the environment. In fact, in a moist environment an oocyst can survive for more than a year (Mai et al., 2009).

Life cycle in intermediate hosts

Intermediate hosts become infected mainly through ingestion of sporulated oocysts or bradyzoites. In accordance with *T. gondii*'s prey-predator way of life, oral infection with bradyzoites has shown less infective to mice than ingestion of oocysts. Just as in cats, *T. gondii* can also pass the placenta and infect the foetus in a pregnant intermediate host (Dubey et al., 2009).

Within the intermediate host, *T. gondii* reproduces strictly asexually. After ingestion of oocysts, the oocyst wall breaks down and sporozoites are free to penetrate the intestinal epithelium, where they differentiate into tachyzoites. Apart from rapid replication, the tachyzoites have the ability to replicate in any kind of cell and therefore spread within the intermediate host. Tissue cysts develop as tachyzoites convert to bradyzoites and the cysts have a high affinity for muscular and neural tissue. Within the cyst the bradyzoites replicate slowly. The lifecycle of *T. gondii* is completed when an infected intermediate host becomes a cat's prey (Dubey et al., 2009).

Infection may also result from ingestion of a large enough number of bradyzoites, following ingestion of tissue cysts in meat from another intermediate host. After the bradyzoites have been released inside the gut, they will infect intestinal enterocytes and differentiate to tachyzoites, where after the process will be identical to that after ingestion of oocytes (Dubey et al., 2009).

Life cycle summarized

Toxoplasma gondii is not solely dependent on its ideal prey-predator life cycle for transmission. Oocysts shed by an infected definitive host will contaminate the environment, where it may infect an intermediate host. If the intermediate host is an animal that can become prey to a felid, the life cycle of *T. gondii* can be completed. This is the most direct and effective way of transmission but it is also possible for the parasite to be transmitted between definitive hosts without passing by an intermediate host, although this route of infection is less effective. Also, if an infected intermediate host is not a potential prey for a felid, *T. gondii* can still infect another intermediate host via carnivorism or pass over the placenta to a foetus (Dubey et al., 2009).

Transmission

Cats and other felids are present everywhere, both in the wild and in close proximity to humans as domestic pets. Due to cat faeces being dispersed everywhere, infective oocysts can be present anywhere in the outside environment - soil, water, animal feed, contaminated human food such as berries, salad, etcetera. This is the main infectious route for intermediate hosts (Dubey et al, 2009).

The main way of horizontal infection in humans is either through ingestion of under-cooked meat with tissue cysts or via oocyst contaminated soil, water or food. A potential cause of infection is directly from cat faeces, most likely from cleaning a litter box, though the risk for transmission this way has been estimated as low. Transmission is also possible through transplanting of cyst-containing organs, blood transfusions or even injections. Another way of transmission, that may have fatal consequences, is vertical infection. A primary infection in a pregnant woman can lead to tachyzoite-induced infection of the foetus, potentially causing abortion or serious foetal infection (Tenter et al, 2000).

Horses seem to be highly resistant to infection by *T. gondii*. Ingestion of sporulated oocysts from the environment is the main way horses become infected with *T. gondii*, by grazing contaminated soil or contaminated feedstuff (Dubey et al., 2009). Some studies have presented results suggesting that there is possible transplacental infection in equines (Turner and Savva, 1991; Turner and Savva, 1992).

Consequences of infection

The widespread fact is that infection with *T. gondii* usually does not produce clinical signs in definitive hosts nor intermediate hosts. However, a review by McAllister (2005) puts this to question as several published studies and case-reports implies that infections do occur in immune-competent humans.

Subclinical infections are typical for felines and if symptoms occur it is usually seen in congenitally infected kittens or cats with suppressed immune system. Clinical signs in felines with acute toxoplasmosis are fever, ocular inflammation, anorexia, lethargy, abdominal discomfort and neurological abnormalities (Dubey et al., 2009).

In humans, the two groups of people in risk of developing clinical toxoplasmosis are pregnant women and immune-incompetent individuals. Congenital toxoplasmosis may cause abortion, death or abnormalities of the foetus. Symptoms of infection in immunocompetent humans can be lymphadenopathy or eye infections. In immune-incompetent humans infections have caused encephalitis, sepsis, myocarditis etcetera. (Tenter et al., 2000). In later years, connections between neuropsychiatric disease and toxoplasmosis have been made in several studies, as summarized in the review by McAllister (2005).

Clinical toxoplasmosis has not yet been diagnosed in horses (Dubey et al, 2009). There is however reports of possible eye-infections in horses due to *T. gondii*, but these results have not been proven (Turner and Savva, 1990). Abortion in horses or neurological disease have been discussed but not demonstrated.

Diagnosis

Methods used to diagnose toxoplasmosis are histology, immunohistochemical staining, PCR (polymerase chain reaction) and serology. The serological tests listed are plentiful; dye test (DT), indirect hemagglutination test (IHA), complement fixation test (CF), modified agglutination test (MAT), latex agglutination test (LA), indirect fluorescent antibody test (IFAT), enzyme-linked immunoabsorbent assay (ELISA) and western blotting (Dubey et al., 2009). Most studies of prevalence in horses use IFAT for analysis, with a cut-off value of 1:64 (for references, see table 1). Camossi et al. (2006) used a cut-off value of 1:16.

Serologic cross-reactivity between *T. gondii* and *Neospora* spp. is not considered a problem (Trees et al., 1993).

Neospora

Neospora spp. are closely related to *Toxoplasma gondii* and was not described as a separate genus until 1988 (Dubey et al., 1988). Due to its late discovery some aspects of the parasite is still inconclusive (Dubey and Schares, 2011). *Neospora caninum* is considered the type species of the genus, but a separate species named *Neospora hughesi* has been found in horses (Marsh et al., 1998). In short of a few important exceptions, *N. caninum* is very similar to *T. gondii* both structurally and in its life cycle.

Hosts

Definitive hosts of *N. caninum* are canids. Viable oocysts have so far been found in faeces from domestic dogs, Australian dingoes, coyotes and gray wolves (Dubey et al., 2011; Gondim et al., 2004; King et al. 2010; McAllister et al., 1998). As for *Toxoplasma gondii* many different mammals have been found having antibodies against *Neospora caninum*, but according to Dubey and Schares (2011) *Neospora* spp. has only been isolated from cattle, sheep, water buffalo, dog, horse, bison and white-tailed deer. According to McCann et al. (2008), humans do not act as intermediate hosts for *Neospora* spp.

In contrast to *N. caninum*, *N. hughesi* has only been isolated from horses (Marsh et al., 1998). There is no knowledge yet regarding definite and intermediate hosts (Hoane et al., 2006).

Transmission and life cycle

The life cycle of *N. hughesi* is not known (Hoane et al., 2006), but the life cycle of *N. caninum* is generally the same as that of *T. gondii*. The three infectious stages of the parasite are tachyzoites, bradyzoites (tissue cysts) and sporozoites (sporulated oocysts). Canines or coyotes become infected by ingestion of meat containing tissue cyst, where after the parasite reproduces sexually inside the host and is excreted as non-sporulated oocysts in the faeces. *N. caninum* oocysts are as resistant in the environment as oocysts of *T. gondii* (Dubey and Schares, 2011). Both transmission between definitive hosts via oocysts in faeces or vertical transmission, either by transmission over the placenta or by infection of puppies through the milk, is possible but is not thought to be an important way of infection (Barber and Trees, 1997).

The research regarding the life cycle and transmission in intermediate hosts of *Neospora* spp. is inconclusive. It is likely that they, much like in toxoplasmosis, become infected through ingestion of oocysts in feed, water or soil (Dubey and Schares, 2011).

In cattle, the species where *N. caninum* infections are known to cause most problems, vertical transmission have been proven an important route for infection and occur in two different ways. Exogenous transmission is seen when a pregnant cow becomes infected through ingestion of oocysts, while endogenous transmission happens when the bradyzoites in a chronically infected cow becomes reactivated during pregnancy (Williams et al., 2009).

The research is not conclusive regarding transplacental infection in horses. The results from a study by Antonello et al. (2012) suggests that although post-natal infections are most common, vertical infection may be important. Several studies have shown evidence of intra-uterine infection of *Neospora* spp. by significantly high titres of antibodies in pre-suckling foals (Antonello et al., 2012; Pusterla et al., 2011; Veronesi et al., 2008), an analytic method made possible by the fact that the placenta in horses do not allow transfer of antibodies to the fetus (Abd-Elnaeim et al., 2006).

Consequences of infection

Clinical neosporosis in dogs is mainly seen in puppies, but can affect animals of any age. Affected older dogs are often under treatment with immune-suppressants when becoming ill. Infected puppies usually does not show signs of sickness until an age of three weeks or older. Clinical signs are hindlimb paresis and paralysis, weakness in forelimbs and swallowing and breathing difficulties. The course of the disease can be peracute or chronic, but always ends in either death or euthanasia in the absence of treatment. Clinical signs in older dogs are not as consistent (Lyon, 2010).

In cattle, neosporosis is a well-known cause of abortion with infected cows aborting from 3 months of pregnancy until time for parturition, but is usually seen in mid-gestation. Neosporosis in cattle can become economically challenging as outbreaks can occur in epidemic herds, with sometimes more than half the herd may abort within a few weeks. Abortion is the only clinical signs reported in adult cows, but neurological and other defects have been reported in young calves (Dubey and Lindsay, 1996).

As with other areas of the protozoa *Neospora*, there are many questions left to answer about clinical neosporosis in horses. It is believed that *N. hughesi* can cause Equine Protozoal Myeloencephalitis (EPM), a progressive degenerative neurological disease of the central nervous system quite common in the United States. The EPM is a debilitating disease where treatment early when the symptoms are mild might ensure a recovery, but more severely affected horses carry a poor prognosis. The disease has been known to be caused only by *Sarcocystis neurona*, another protozoan, but lately several case reports from North America have been published where *Neospora* spp. has been found in horses diagnosed with EPM (Finno et al., 2010; Marsh et al., 1996; Wobeser et al., 2009). A study by Kligler et al. (2007) in Israel found positive correlation between antibody titer against *Neospora* spp. and neurological disease. It is not known whether only *N. hughesi* alone can cause EPM, or if *N. caninum* has the ability as well.

Several studies have been made to establish whether neosporosis is a cause of abortions in equines and although not fully proven, evidence suggests that infection may have a role in reproductive problems in mares. One study by Villalobos et al. (2006) did find a positive association between antibody-titer against *Neospora* spp. in mares with abortion in late gestation, as well as studies by Pitel et al. (2003) and Kligler et al. (2007). In another study by Dubey & Porterfield (1990) tachyzoites were described in the lungs of an aborted 9 month old foetus. Several other studies have failed to show any correlations between neosporosis and abortions in equines (McDole et al., 2002; Veronesi et al., 2008). In a study by Pusterla et al. (2011), that showed evidence for in-utero infection of foals by *Neospora* spp., they did not see any short-term evidence of clinical signs in foals with high pre-colostral antibody titers.

Diagnosis

Methods used to diagnose neosporosis are histology, immunohistochemical staining, PCR (polymerase chain reaction) and serology. For detection of infection in living animals serology is used, the method can however not tell when infection occurred (Dubey & Schares, 2006). Techniques used for detection of antibodies in blood are the indirect fluorescent antibody test (IFAT), immunoblotting (IB), direct or modified agglutination test (DAT or MAT) and enzyme-linked immunosorbent assays (ELISAs). IFAT is generally approved as the “gold standard” and is used in many of the serological studies made on *Neospora* spp. (Björkman and Uggla, 1999).

The cut-off titer usually employed when performing serology on equine blood samples is 1:50 (for references, see table 3).

Since the research on neosporosis in horses is sparse, knowledge about the antibody response has to be extrapolated from research on dogs. When exposure occurs, seroconversion in dogs usually happens after two to three weeks (McAllister et al., 1998). Important to remember when performing serology is that positive results, i.e. detection of antibodies above cut-off value, only show exposure and not necessarily disease. In a study by Barber and Trees (1996) they found that most clinically healthy dogs had a titer equal to or below 1:800. Clinically affected dogs do not however necessarily need to have a high titer. The mule in the study by

Finno et al. (2010), diagnosed with EPM due to neosporosis, had a titer of 1:160 (IFAT, cut-off value 1:50).

Gondim et al. (2009) proved that there is cross-reactivity between *N. caninum* and *N. hughesi*. After exposure to *N. caninum*, all animals (previously negative) had a positive antibody titer against both *N. caninum* and *N. hughesi*, with the only difference that titers against *N. hughesi* mostly were one dilution lower. This means that it is not possible today to distinguish between the two species with serology methods.

Seroprevalence of *Toxoplasma gondii* and *Neospora* spp. in horses in Brazil

The information published about the seroprevalence of anti-*Toxoplasma gondii* antibodies in horses in Brazil is sparse and several of these articles are available only in Portuguese and are difficult to find. Results from available published studies are summarized in table 1. End-point antibody titers from published studies are summarized in table 2.

Table 1. *Seroprevalence of Toxoplasma gondii in equines in Brazil from published studies*

Overall prevalence	Type of equine	States	Analytic method and cut-off value	Reference
15.8% (16/101)	Thoroughbreds in training facilities	Sao Paulo, Rio de Janeiro and Rio Grande do Sul	MAT	Dubey et al., 1999
12.1% (21/173)	Not specified	Paraná	IFAT 1:64	Garcia et al., 1999
7.0% (138/1984)	Not specified	Not specified	MAT	Langoni et al., 2007
5.9% (15/253)	Not specified	Botucatu, Sao Paulo state	IFAT 1:16	Camossi et al., 2010
11.6% (46/398)	Horses at slaughterhouse	Paraná, Minas Gerais, Rio de Janeiro, Goiás, Mato Grosso do Sul and Mato Grosso	IFAT 1:64	Evers et al. 2013
23.8% (94/395) ³ 43.2% (38/88) ⁴	Mules and donkeys ²	Pernambuco, Rio Grande do Norte, Paraíba and Sergipe	IFAT 1:64	de Oliveira et al., 2013
17.0% (17/100)	Cart horses ¹	Metropolitan area of the city Curitiba	IFAT 1:64	Finger et al., 2013

¹ Horses used for labour in the urban area.

² Type of usage not specified.

³ Mules.

⁴ Donkeys.

Table 2. *End-point antibody titers of Toxoplasma gondii in equines in Brazil from published studies*

Reference	Maximum titer	No. of horses with end-point titer 1:64 (%)
Garcia et al. (1999)	1:256	57.1% (12/21)
Finger et al. (2013)	1:64	17.0% (17/100)

Similar to *Toxoplasma gondii*, there are few studies regarding the seroprevalence of *Neospora* spp. in equines in Brazil. Most of these studies are represented in table 3. End-point antibody titers from published studies are summarized in table 4.

Table 3. *Seroprevalence of Neospora spp. in equines in Brazil from published studies*

Overall prevalence	Type of equine	States	Analytic method and cut-off value	Reference
0% (0/101)	Thoroughbreds in training facilities	Sao Paulo, Rio de Janeiro and Rio Grande do Sul	MAT	Dubey et al., 1999
2,5% (24/961)	Farm horses and horses sent to slaughter	From ten different states	ELISA	Hoane et al., 2006
10.3% (114/1106) ² 15.4% (77/550) ³ 6.1% (37/606) ⁴	Various	Sao Paulo	IFAT 1:50	Villalobos et al., 2006
15.9% (34/214)	Cart horses ¹ and Crioula breed horses	Rio Grande Sul	IFAT 1:50	Toscan et al., 2011
14.4% (14/97)	Cart horses ¹	Urban areas of the city Curitiba	IFAT 1:50	Villalobos et al., 2011
4.1% (25/615)	Various	Mountain and coastal region of Santa Catarina	IFAT 1:50	de Moura et al, 2013

¹ Horses used for labour in the urban area.

² The total frequency in the study.

³ Samples sent in for analysis of Equine Herpes Virus-1 (case group including mares with reproductive problems and males in close contacts with mares with reproductive problems).

⁴ Samples sent in for diagnostic work-up Equine Infectious Anemia (control group declared healthy by veterinarian).

Table 4. *End-point antibody titers of Neospora spp. in equines in Brazil from published studies*

Reference	Maximum titer	No. of horses with end-point titer 1:50 (%)
Villalobos et al. (2006)	1:400	9/37 (24.3%) ¹ ; 45/77 (58.4%) ²
Villalobos et al. (2012)	1:200	6/14 (42.8)
De Moura et al. (2013)	1:400	13/25 (52)

¹ Blood samples sent in for diagnostic work-up Equine Infectious Anemia (control group declared healthy by veterinarian).

² Blood samples sent in for analysis of Equine Herpes Virus-1 (case group including mares with reproductive problems and males in close contacts with mares with reproductive problems).

Horse categories in the state of Pará

Farm horses

The majority of the horses in Pará state are kept on farms (fig. 1) and used for routine services such as cattle management (fig. 2) and transportation in rural communities, but also for breeding. The horses are usually either born at the farm or bought from other farms in the surrounding area. They are kept outside all year round, generally on extensive areas of land together with other farm animals. Typical breeds are mixed breeds, mules or pure donkeys, even though pure horse breeds such as Quarter horses do occur.



Fig. 1. Farm donkey. Photo: Emma Norlander.



Fig. 2. Farm horse. Photo: Emma Norlander.

Urban horses

The expression urban horse (fig. 3), a.k.a. cart horse, is assigned to horses that are used for labour within the urban area. These horses work up to 12 hours per day pulling a cart, carrying out tasks such as transportation of people and goods and collecting waste for recycling. They are mostly kept at the property of the owner, i.e. in the backyard, or on meadows within the city. Common breeds are smaller mixed breeds, mules or donkeys. The owners of this type of horses are economically challenged and therefore the number of urban horses in the cities declines as general living standards in Brazil increases, which explains the small number of urban horses present in some of the cities included in the study. These horses are often of poor nutritional status and lack the possibility of veterinary care.



Fig. 3. Urban mule.

Photo: Emma Norlander.



Fig. 4. Sport horse.

Photo: Emma Norlander.

Horses used for sport

Sport horses (fig. 4) in Pará are mainly used for vaquejada, a traditional cowboy competition that is popular in the northeast of Brazil involving the catching of a calf. The horses are either kept at farms, where they also can be used for cattle management, or in stables in the urban area. Horses used for sports are quarter horses, pure or part breed. These horses have considerable economical value, especially those who perform well in competitions, and are generally kept in good condition. The sport horses travel, some times considerable distances, to attend competitions and are kept together with unknown horses from other areas during these events. As they participate in competitions, they travel a lot between cities and are kept in a way that make them potential sources of infectious disease.

MATERIAL AND METHODS

Site of sample collection

Sample collection was performed in three different cities in Pará state of Brazil - Brasil Novo, Óbidos and Santarém (fig. 5 and 6).

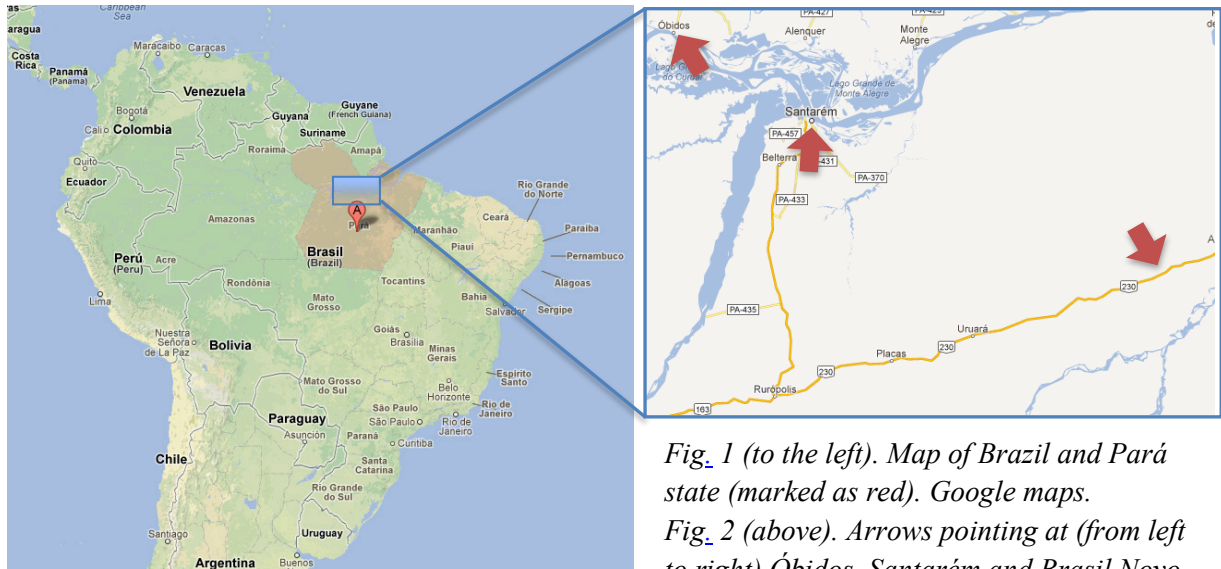


Fig. 1 (to the left). Map of Brazil and Pará state (marked as red). Google maps.

Fig. 2 (above). Arrows pointing at (from left to right) Óbidos, Santarém and Brasil Novo. Google Maps.

Two of the cities, Óbidos and Santarém are situated nearby water. Óbidos lies by the Amazon River while Santarém resides at the right bank of the Tapajós River (fig. 7), close to where the Tapajós joins the Amazon. Brasil Novo, on the other hand, is located along the Trans-Amazonian highway (fig. 8). The cities also differ in size. While Santarém is one of the largest and most important cities in the state, Óbidos and Brasil Novo are smaller both in size and population.



*Fig. 7. Tapajós River.
Photo: Emelie Andersson.*



*Fig. 8. Trans-Amazonian Highway.
Photo: Emma Norlander.*

Study design

Population definition

The animals included in the study were divided into three different groups of equids categorized into farm horses, urban horses and horses used for sport. There were no exclusion criteria such as age, gender or health status. The study was not limited to horses, as both mules and pure donkeys were accepted. To simplify, the term “horses” will be used in the remainder of this report, including all the types of equids mentioned above. For each horse information about age, sex and breed was collected.

Sampling method

Cluster sampling was used when sampling the farm horses, i.e. the farms were selected by simple random sampling and all the horses present at the farm were included in the study. The Brazilian Institute of Geography and Statistics, Municipal Agricultural Production, was the source for information about the number of farms and horses in each city. Due to the inaccessibility of large parts of the rainforest, it was not possible to inform the farm owners prior to the sampling. Therefore, some farms did not have all horses present as sampling was performed during the season for “varzea”; a specific type of riverbed pasture usually located a considerable distance from the farm. In these cases, only the horses present at the farm were sampled. If no horses were present in the selected farms, due to varzea or other reasons or if the farm did not have any horses, the closest neighbouring farm was selected instead. This solution was also applied if the farm owner declined to participate in the study.

Since there are no public records kept of the urban horses in either city, the intention was to include all of the urban horses present in the study and the method chosen to locate them was by investigations at site. In Brasil Novo there was a rallying point where the urban horses were stationed during the day. In the other cities urban horses were discovered by sight or localised with the assistance of other horse owners.

The sport horses in this study were primarily selected from an occurring sports event during the time for sample collection in each city. At these events every horse that the owners allowed to be sampled were included. In Brasil Novo no event took place during the time period for fieldwork, whereby sport horses kept at a sports arena in the city area were collected instead.

Sample size

A total number of 440 horses were included in this study. The distribution of samples between the cities and between the different categories of horses is shown in Table 5. Number of farms and farm horses sampled is specified in Table 6.

Table 5. *Number of horses sampled in the cities of Óbidos, Brasil Novo and Santarém in the state of Pará, Brazil*

City	Farm horses	Urban horses	Sport horses	Total no. of horses
Óbidos	95	3	8	106
Brasil Novo	146	6	13	165
Santarém	68	82	19	169
Total	309	91	40	440

Table 6. *Total number of farms and horses in each city, number of farms included in the study and number of horses sampled*

City	Total no. of farms with horses	Total no. of horses	No. of farms sampled	No. of horses sampled
Óbidos	980	4975	9	95
Brasil Novo	1135	3751	11	146
Santarém	648	2836	6	68
Total	2763	11562	26	309

Sample collection

Sample collection took place between August 2011 and October 2012, though the majority of samples were collected during September 2012. For each horse included in this study blood was sampled aseptically through the jugular vein into evacuated tubes (fig. 9). One sample tube without anti-coagulant was collected from each individual and within 24 hours the tubes were centrifuged at 2000 g for 20 minutes, until separation of serum occurred. The serum was identified, aliquoted into Eppendorf tubes and kept at -20° C until analysis. The samples were transported to University of São Paulo for analysis.



Fig 9. *Sampling of blood. Photo: Emelie Andersson.*

Laboratory technique for antibody detection

Cultivation of tachyzoites and preparation of slides

Multiwell Teflon-coated glass slides coated with tachyzoites were provided by the Parasitic Diseases Laboratory of the Faculty of Veterinary Medicine and Zootechny, University of São Paulo. The tachyzoites as well as the slides had been cultivated and prepared by the laboratory.

Toxoplasma serology

For detection of anti-*Toxoplasma gondii* antibodies IFAT was used according to Dubey et al. (1999). The cut-off value chosen for *Toxoplasma gondii* was 1:16, i.e. titres equal to or greater than 1:16 were considered positive (Camossi et al., 2010).

Qualitative testing

The serum samples were diluted to 1:16 in a sterile saline solution buffered by phosphate (PBS). The concentrated solution had a pH of 7.2 and was prepared from 0.731 M NaCl, 0.027 M KCl, 0.105 M Na₂HPO₄ and 0.018 M KH₂PO₄, with distilled H₂O added until 1000 ml. The PBS solution used was made by diluting the concentrated PBS to 1:10 with distilled water. 20 µl of diluted serum was placed in each well of the microscope slides. All slides prepared included two wells of standard positive and negative control sera from horse. After incubation for 30 minutes at 37°C in a humid chamber, the slides were washed three times for 10 minutes in the PBS. After washing the slides were allowed to dry at room temperature, before 20 µl of horse anti-IgG conjugate (conjugated with fluorescein isothiocyanate) and Evan's blue was added to each well. The slides were incubated anew for 30 minutes and then washed according to the same regimen as described previously. To avoid decreased intensity of the fluorescence, the slides were kept in the dark during both washing and when allowed to dry at room temperature.

Quantitative testing

Samples considered positive in the trials were titrated. The sera were diluted in PBS in twofold titrations, starting at 1:16 (making the next titer 1:32, 1:64, 1:128 and so on). The samples were titrated until reaching an end-point titer, i.e. a maximum titer for reaction. Production of the slides was done according to the same principles as in the trials.

Neospora serology

For detection of anti-*Neospora* spp. antibodies IFAT was used according to Dubey et al. (1988). Cut-off value for *Neospora* spp. was set to 1:50 (Dubey et al., 1999). For detection of antibodies to *Neospora* spp., IFAT against *Neospora caninum* strain NC-1 was used.

Qualitative testing

The principles of IFAT for *Neospora* are in general the same as described for *Toxoplasma gondii*, with a few differences. The serum samples are diluted to 1:50 and with a saline solution buffered in phosphate. The *Neospora* PBS for serum-dilution had a pH of 7.2 and contained 0.0084 M Na₂HPO₄, 0.0018 M Na₂PO₄ 0.146 M NaCl and 1% bovine serum antigen (BSA), with distilled H₂O added to 1000 ml. The washing regimen is 3 times for 5

minutes and the washing buffer was different from the serum-dilution buffer. The washing buffer had a PH of 9.0 and was prepared as a concentrate containing 0.108 M Na₂CO₃, 0.4 M NaHCO₃, 0.145 M NaCl and distilled H₂O to 1000 ml. To obtain washing PBS for usage, the concentrate is diluted with distilled H₂O to 1:4.

Quantitative testing

See titration for *Toxoplasma gondii*, with the exception of doubling titration starting at 1:50 (with the following titres being 1:100, 1:200 and so on).

Visualization and interpretation

For visualization in microscope, buffered glycerine (pH 8.0) and a cover slip was applied on each slide. Diagnosis was made with a fluorescent microscope at 40x. Positive results were diagnosed by observation of tachyzoites with complete peripheral fluorescence in most visual fields. Samples with fields containing more than a few tachyzoites showing partial peripheral fluorescence, apical fluorescence and/or non-fluorescent tachyzoites were considered negative.

Statistical analysis

To investigate the relationship between different determinants, Chi-square test was used and statistical significance was set to $p < 0.05$.

RESULTS

Toxoplasma gondii prevalence and titers

Table 7 summarises the results of the indirect immunofluorescence (IFA) for *Toxoplasma gondii*. Antibodies were found in all categories of horses and in each municipality, with the exception of urban horses in Óbidos and Brasil Novo where serum levels of antibodies were absent or below cut-off value. The over-all frequency of occurrence of anti-*Toxoplasma gondii* antibodies was 6.4% (28/440). Within the cities the highest percentage of prevalence was found in Óbidos, where 10.4% (11/106) of the horses were positive. 15% (6/40) of the sport horses had titers against *Toxoplasma gondii* and thereby the highest percentage of prevalence of the three horse categories.

Table 7. Results from indirect immunofluorescence assay (IFA) for *Toxoplasma gondii* in horses of different categories from the cities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

City	Horse category							
	Farm horses		Urban horses		Sport horses		Total	
	n*	Reactive sera (%)	n*	Reactive sera (%)	n*	Reactive sera (%)	n*	Reactive sera (%)
Óbidos	95	10 (10.5)	3	0 (0)	8	1 (12.5)	106	11 (10.4)
Brasil Novo	146	7 (4.8)	6	0 (0)	13	3 (23.1)	165	10 (6.1)
Santarém	68	2 (2.9)	82	3 (3.7)	19	2 (10.5)	169	7 (4.1)
Total	309	19 (6.1)	91	3 (3.3)	40	6 (15.0)	440	28 (6.4)

* Number of sera tested.

The distribution of end-point antibody titers against *Toxoplasma gondii* is shown in Table 8. The highest end-point titer seen was 1:1024 and was found in a serum sample from Óbidos. End-point titers in Óbidos are mainly distributed in the lower spectra, where as in Santarém all but one of the samples has an end-point titer of 1:128.

Table 8. IFA end-point antibody titers for *Toxoplasma gondii* in horses from the cities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

City	n*	End-point antibody titer						
		1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Óbidos (%)	11	7 (63.6)	2 (18.2)	-	-	1 (9.1)	-	1 (9.1)
Brasil Novo (%)	10	2 (20.0)	3 (30.0)	1 (10.0)	3 (30.0)	1 (10.0)	-	-
Santarém (%)	7	-	-	-	6 (85.7)	-	1 (14.3)	-
Total	28	9 (32.1)	5 (17.9)	1 (3.6)	9 (32.1)	2 (7.1)	1 (3.6)	1 (3.6)

* Number of positive sera.

Neospora prevalence and titers

Table 9 shows the results of the IFA for *Neospora* spp. The over-all frequency of occurrence of anti-*Neospora* spp. antibodies was 4.5% (20/440). Antibodies were found in samples from all categories of horses in Santarém, whereas in Brasil Novo all horses sampled were negative

for anti-Neospora antibodies (serum titer ≥ 50). In Óbidos reactive sera was found in farm and sport horses, but not in urban horses. Of Óbidos and Santarém, the latter had the highest percentage of prevalence at 9.5% (16/169). Within the different horse categories, urban horses had the highest prevalence at 13.2% (12/91).

Table 9. Results from indirect immunofluorescence assay (IFA) for *Neospora* spp. in horses of different categories from the cities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

City	Horse category							
	Farm horses		Urban horses		Sport horses		Total	
	n*	Reactive sera (%)	n*	Reactive sera (%)	n*	Reactive sera (%)	n*	Reactive sera (%)
Óbidos	95	3 (3.2)	3	0 (0)	8	1 (12.5)	106	4 (3.8)
Brasil Novo	146	0 (0)	6	0 (0)	13	0 (0)	165	0 (0)
Santarém	68	1 (1.5)	82	12 (14.6)	19	3 (15.8)	169	16 (9.5)
Total	309	4 (1.3)	91	12 (13.2)	40	4 (0.1)	440	20 (4.5)

* Number of sera tested.

The distribution of end-point antibody titers against *Neospora* spp. is shown in Table 10. The highest end-point titers are seen in Santarém where a majority of the titers are equal to or greater than 1:800, the maximum end-point titer being 1:12800. The highest titers were solely found in urban horses. The horse with the titer 1:50 was the only farm horse positive in Santarém. The titer 1:200 was found in 1 sport horse and 2 urban horses. The 5 horses that had a titer of 1:800 were 2 sport horses and 3 urban horses. All horses with titers of 1:3200 and 1:12800 were urban horses. In Óbidos, no serum sample had a titer higher than 1:200.

Table 10. IFA end-point antibody titers for *Neospora* spp. in horses from the cities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

City	n*	End-point antibody titer								
		1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800
Óbidos (%)	4	-	1 (25.0)	3 (75.0)	-	-	-	-	-	-
Brasil Novo (%)	0	-	-	-	-	-	-	-	-	-
Santarém (%)	16	1 (6.3)	-	3 (18.8)	-	5 (31.3)	-	5 (31.3)	-	2 (12.5)
Total	20	1 (5.0)	1 (5.0)	6 (30.0)	-	5 (25.0)	-	5 (25.0)	-	2 (10.0)

* Number of positive sera.

Prevalence on the basis of determinants

As seen in table 11, there was no statistical significant difference between seropositive males and females in the *Toxoplasma gondii* group ($p > 0.05$). When analysing antibodies against

Neospora spp., the seroprevalence was significantly higher in males compared to females ($p \approx 0.0275$).

Neither the determinant breed nor age had any statistically significant association to increased prevalence of *Toxoplasma gondii* or Neospora spp. ($p > 0.05$).

There was a significant difference for the determinant horse category for both *Toxoplasma gondii* ($p \approx 0.0394$) and Neospora spp. ($p \approx 0.0000$). When performing chi-square analysis within the determinant group for *Toxoplasma gondii*, only the prevalence in sport horses was significantly higher than the prevalence in urban horses ($p \approx 0.0390$ with Yate's correction for continuity employed, since at least 20% of the expected frequencies were lower than 5). For Neospora spp., only the prevalence in urban horses was significantly higher than the prevalence in farm horses ($p \approx 0.0000$ with Yate's correction for continuity employed, since at least 20% of the expected frequencies were lower than 5).

When calculating the association between seroprevalence and municipality, there was no significant association for *Toxoplasma gondii* ($p > 0.05$). Neospora spp. had a significant difference between seroprevalence in the different municipalities ($p \approx 0.0000$). Performing chi-square testing within the determinant, the seroprevalence of Neospora spp. was significantly higher in Obidós than in Brasil Novo ($p \approx 0.0457$ with Yate's correction for continuity employed, since at least 20% of the expected frequencies were lower than 5). The frequency was also higher in Santarém than in Brasil Novo ($p \approx 0.0001$).

Table 11. Seroprevalence of anti-*Toxoplasma gondii* and anti-*Neospora spp.* antibodies according to the determinants gender, breed, age, horse category and municipality

		<i>Toxoplasma gondii</i>		Neospora spp.	
Determinants	Total no.	No. (%)	p ¹	No. (%)	p ¹
Gender					
Male	271	14 (5.2)	0.1925	17 (6.3)	0.0275
Female	169	14 (8.3)		3 (1.8)	
Breed					
SRD ⁴	280	23 (8.2)	0.0992	14 (5.0)	0.5947
QM ⁵	131	5 (3.8)		5 (3.8)	
Donkey	6	0 (0) ²		0 (0) ²	
Mule	9	0 (0) ²		1 (11.1) ²	
Other ⁶	14	0 (0) ²		0 (0) ²	
Age					
< 1	17	0 (0)	0,6515 ³	0 (0) ²	0.0849
1-5	170	13 (7.6)		3 (1.8)	
6-10	188	9 (4.8)		12 (6.4)	
≥ 11	65	6 (9.2)		4 (6.2)	
Horse category					
Farm	309	19 (6.1)	0.0394	4 (1.3)	0.0000 ³
Urban	91	3 (3.3)		12 (13.2)	
Sport	40	6 (15.0)		4 (10.0)	
Municipality					
Óbidos	106	11 (10.4)	0.1170	4 (3.8)	0.0000
Brasil Novo	165	10 (6.1)		0 (0)	
Santarém	169	7 (4.1)		16 (9.5)	

¹ p-value from chi-square test.

² Frequency below 1, chi-square test not performed.

³ At least 20% of expected frequencies less than 5, Yate's correction for continuity employed.

⁴ Sem Raça Definida, meaning non-defined mixed breed. Definition of a type of horse commonly found in Pará state.

⁵ Quartamila, aka. Quarter horse. Includes pure breed as well as mixes of Quarter horse.

⁶ Breeds too few to create a separate breed category; Mangalarga/Paulista, Paint horse and Mestiça.

DISCUSSION

Seroprevalence

The true seroprevalence of *Toxoplasma gondii* and *Neospora* spp. in horses in Brazil is unknown. Since this is the first study investigating the seroprevalence of the both genus of protozoa among horses in the north of Brazil (including the states Pará, Amazonas, Tocantins, Rondônia, Acre, Roraima and Amapá), it is interesting to see that the frequency of seropositive horses are rather low for both pathogens. An assumption of higher frequencies could easily have been made when taking in to account the generally poor economical status of the people in this area and the type of housing of horses common here. Where horses are kept in close contact with stray cats and dogs as in the cities or on extensive grazing amongst wild and domestic canids and felids, one might assume that exposure to oocysts would be high. Both pathogens are despite this in the lower end of the frequencies reported in earlier studies from other areas of Brazil (tables 1 and 3).

This study describes a frequency of anti-*Toxoplasma gondii* antibodies in horses of 6.4% (28/440). Compared to other studies made investigating the seroprevalence among horses in Brazil, this is a rather low frequency. The prevalence is similar to frequencies of 7.0% (Langoni et al., 2007) and 5.9% (Camossi et al., 2010) found in two other studies. The material in the study by Camossi et al. (2010) consisted of blood samples sent in to a laboratory to be analysed for Equine Infectious Anemia (EIA). This implies that the owners of the sampled horses had economical possibility to provide veterinary care and might suggest that the horses were kept in a different way than many of the horses in our study. This could have made them less exposed to oocysts and also in better condition. The study by Camossi et al. (2010) is though the only study listed in table 1 that, like this study, used an IFAT cut-off value of 1:16. Langoni et al. (2007) also used blood samples sent in to a laboratory to be analysed for Equine Infectious Anemia (EIA), but used MAT instead of IFAT for analysing the samples. Most studies reports frequencies ranging between 11.6-17.0% (se table 1). Some of these studies might not be suitable for comparison of frequency. Two of the studies only sampled one breed or one category of horses (thoroughbreds respectively cart horses) and had a small sample size (Dubey et al. 1999; Finger et al, 2013).

As a lower cut-off value for IFA for *Toxoplasma gondii* was chosen for this study compared to many other studies made in horses in Brazil, i.e. 1:16 instead of 1:64, this has to be kept in mind when comparing frequencies. A lower cut-off value has the advantage of less false negative, but at the same time the risk of false positive individuals increases. Of the studies listed in table 1, only the study by Camossi et al. (2010) used the same cut-off value and can be used for comparison. When excluding individuals with titers of 1:16 and 1:32, this study shows a seroprevalence of 3.2% (14/440). This prevalence is lower than any of the other prevalences listed in table 1.

The overall seroprevalence of *Neospora* spp. among horses in this study was 4.5% (20/440). This result is similar to the seroprevalence found in the studies by Hoane et al. (2006) and de Moura et al. (2013), where the results were 2.5% (24/961) and 4.1% (25/615) respectively. Both of the compared studies sampled horses from different geographical areas as well as different categories of horses, resembling the conditions of the present study. To take into

account is the fact that the study by Villalobos et al. (2006) used MAT instead of IFAT to detect antibodies. The study published by Dubey et al. (1999) did not find any seropositive horses (0/101). A possible explanation to the absence of antibodies could be that all sampled horses were thoroughbreds housed in training facilities and therefore might not have been exposed to oocysts in the same way as other horses. Also the sample size was small. Other studies have presented somewhat higher prevalences – 10.3% (114/1106), 14.4% (14/97) and 15.9% (34/212) (Villalobos et al., 2006; Villalobos et al. 2011; Toscan et al. 2011). Two of these studies mainly sampled cart horses and might, due to this lack of other horse categories represented, not be suited to estimate real prevalence within the specified states. The study by Villalobos et al. (2006) used blood samples sent in to the laboratory, half of them for diagnosis of Equine Herpes Virus-1 (EHV-1) and half for analysis of EIA. The total frequency in the study was 10.3% (114/1106) but when excluding the samples sent for diagnosis of EHV-1 the frequency drops to 5.8% (19/325), which is similar to the result in this study. Diagnosis of EHV-1 in horses are commonly made after reproductive problems in mares, why this might cause a high frequency of anti-Neospora antibodies in this particular group as it is likely that *Neospora* spp. can cause reproductive problems in horses as well.

The proven cross-reactivity for the antigens of *N. caninum* and *N. hughesi* means that antibodies directed at either of the two species of *Neospora* will bind to the *N. caninum* tachyzoites used as antigen in the IFAT. It is therefore not possible to distinguish between horses infected with *N. caninum* and *N. hughesi* in this study or any other study using the IFAT method solely. Not much is known regarding *N. hughesi*, as it so far only has been isolated from horses and the knowledge of the effects of *N. caninum* infection in horses are inconclusive. In most studies of *Neospora* spp. different serology methods has been used to demonstrate exposure to the pathogen and find associations to different effects, such as abortions. When taking the cross-reactivity in to account, there is really no way to tell which of the two species is accountable for what. Cross-reactivity also makes it impossible to make any real assumptions of true prevalence for the two species separately. Therefore, the results in this study can only be interpreted for *Neospora* as a genus.

End-point titers

Toxoplasma gondii end-point antibody titers in this study are generally in the lower end of the spectra, with a few exceptions. 32.1% (9/28) of the positive samples had an antibody end-point titer of 1:16 and the highest end-point titer was 1:1024. Only two horses had a titer greater than 1:256. If the cut-off would have been established at 1:64, 7.1% (1/14) of the positive samples would have had an antibody end-point titer of 1:64 and 64.3% (9/14) 1:128. Unfortunately, there were not a lot of studies to compare with from Brazil. As seen in table 2, there is reason to believe that the titers in this study are similar to results from other studies showing low titers, suggesting chronic infection.

The end-point titers for *Neospora* spp. are relatively high in this study compared to other studies. Only 5.0% (1/20) of the seropositive individuals had an end-point titer of 1:50 and the highest end-point titer in the study was 1:2800. 60% (12/20) of the positive horses had an end-point titer higher than 1:400. Compared to results in other studies (see table 4) the highest end-point titer was considerably higher in this study and the frequency of horses with end-

point titer of 1:50 was very low. Urban horses in Santarém solely contributed to the highest end-point titers of *Neospora* spp. The reasons why the end-point titers were so much higher in this study can only be based on speculations, since the knowledge of *Neospora* spp. infections in horses is sparse and because no information about the animals' health status was included in this study. Lower titers should suggest chronic infection and high titers acute infection. The antibody titer decreases with time in chronic infection, why these horses would have titers in the lower spectra. One possible explanation could be that the exposure to oocysts is high in the urban area of Santarém and the cart horses generally are in poor health, which could lead to high frequency of acute infections. It is also possible that there had been a recently increased number of dogs spreading oocysts, which could lead to an increased number of horses exposed and therefore a higher frequency of horses acutely infected.

Seroprevalence on the basis of determinants

The seroprevalence of *Neospora* spp. was significantly higher in males than females. One possible explanation could be that among the urban horses only 11.5% (10/87) were females. This in combination with the fact that the prevalence in urban horses was significantly higher than in farm horses could be responsible for exposure to *Neospora* spp. seeming more common in males. Another potential theory would be that mares to a larger extent are used for reproduction, making them spend more time in the stable or on pasture. Male horses are possibly used for work, either on farms or in urban areas, which could lead to greater exposure to oocysts. These theories would logically apply to *Toxoplasma gondii* as well, where no significant difference between genders was detected. In the study by de Moura et al. (2013), no significant difference of *Neospora* spp. prevalence was seen between males and females.

No significant difference between breeds were seen for neither *Toxoplasma gondii* nor *Neospora* spp. Very few mules and donkeys were included in the study, making conclusions regarding the prevalence for these species irrelevant. All mules and donkeys were negative for *Toxoplasma gondii* and only one mule was positive for *Neospora* spp. It would have been interesting to have had included more mules and donkeys in this study, since de Oliveira et al. (2013) reported very high prevalences of *T. gondii* in mules (23.8%) and donkeys (43.2%).

Within the different age groups, no difference existed statistically. Noteworthy is that none of the horses younger than one year exhibited antibodies towards any of the protozoans.

Significant difference was found for both *T. gondii* and *Neospora* spp. within the determinant horse category. For *T. gondii*, the prevalence was significantly higher in sport horses than in urban horses. To make any conclusions about this might be inadvisable, since the category sport horse in this study is very undefined. The sport horses in Pará included in this study are usually kept either at a farm or in a stable in a city, which would mean that they either have similar exposure as either a farm horse or an urban horse. However, it is likely that sport horses in the city does not have the same mobility as the urban horses and therefore might be less exposed to oocysts. Sport horses that travels a lot could potentially also have a greater exposure. Initially the plan was to only collect sport horses at competing events, so that the category would consist of sample from horses from different origin and who travels to new

places frequently. Then conclusions about whether travelling could impact prevalence might have been able to be made. As there were few events taking place during sampling it was not possible, why only the sport horses in Óbidos was sampled at a competing event. There was also no information about the total number of sport horses in Pará, making the sample number very haphazard. Because of the poor definition of the group, the small sample size and the lack of randomization the result in this study is an unreliable estimate of the true difference between urban horses and sport horses.

The prevalence of *Neospora* spp. was significantly higher in urban horses than in farm horses. Reasons for this could be due to difference in the dog population in the cities versus on farms. In the cities in Pará dogs run freely and therefore the contamination with faeces is high in the urban areas. This means that if there are dogs infected with *Neospora* spp., the exposure of the horses to oocysts is substantial. The cart horses are very mobile and cover considerably distances travelling all over the cities. This, in combination with grazing by the road during work and on meadows or backyards, makes them highly exposed to possible oocysts. On farms however the dog population is less dense, consisting usually only by the dogs belonging to the farm owner. Horses on farms usually are kept on extensive areas of land, which in combination with the low number of domesticated dogs would make the concentration of oocysts low. Dogs can be assumed to keep near the farm buildings and therefore there would not be a high contamination of faeces in the grazing area. Sources of oocysts on farms could instead be wildlife, dead cattle or placentas from aborted cattle.

It is interesting to notice that while the prevalence of *Neospora* spp. is significantly higher in urban horses than in farm horses, the relation is the opposite for *T. gondii*. The prevalence is numerically higher in farm horses than in urban horses, although not statistically significant. As no information about the cat population is known, no real conclusions can be made upon this. If cats are more prevalent on the countryside than in the city, this could be an explanation.

Within the determinant municipality, there was no statistically significant difference between the different sampled areas for *T. gondii*. *Neospora* spp. titers in Brasil Novo were significantly lower than in both Santarém and Óbidos. In Brasil Novo, no seropositive horses were found. The absence of antibodies against *Neospora* spp. in Brasil Novo could have been influenced by the fact that most of the horses sampled in the city, i.e. 89.7% (95/106), were farm horses. Farm horses generally had a low prevalence in this study, 1.3% (4/309). Because of the uneven number of individuals in the different horse categories, the result may not be trustworthy. This difference could also be attributable to factors such as presence of dogs, cattle or management practices. A review by Dubey et al. (2007) mentions that sporulation and survival of coccidian oocysts benefits from humidity and mild temperatures. This is interesting since Brasil Novo is located in the inland and the other two cities by the Amazon. It could be presumed that the weather would be a little milder and more humid in the cities by water. Because of the similarities, the same would be applicable for *T. gondii*. However, the prevalence for the latter was not significantly lower in Brasil Novo compared to the other cities and not more than slightly numerically lower than the prevalence in Santarém.

Laboratory analysis

In this study two different persons performed IFAT analysis on different samples. This is not ideal since the IFAT results are somewhat subjective. The visual reactions can be interpreted different depending on the individual performing the analysis. Difference in training and experience can also influence interpretation. In this study the persons performing the analysis had the same guidance in the laboratory, which would decrease the risk of differing results.

Additional data

In retrospect, there would have been of great interest to have more information about the individuals included in this study. Data on the dog and cat population on the farms sampled and in the cities, as well as if the horses had any contact with cattle, would have presented the opportunity to evaluate the association with these factors. De Moura et al. (2013) found a significantly higher prevalence of anti-*Neospora* spp. antibodies in horses that had been in contact with dogs or cattle. Information regarding the horses' health status would have allowed a chance to relate titers to health problems such as neurologic disease or reproductive problems. This would have been especially interesting when discussing the end-point titers of *Neospora* spp. as these generally were high, but also to see if there was any association between mares with reproductive problems and seropositivity. Knowledge of relations between the horses could have given the opportunity to investigate whether positive mares had any impact on their offspring regarding seropositivity.

Consequences of neosporosis and toxoplasmosis in horses

There are no solid proof of clinical disease in horses infected with *T. gondii*, why the consequences of infection only are relevant when viewing horses as a potential cause of human toxoplasmosis. The prevalence of *T. gondii* infection in horses is relatively small and the risk of become infected by ingestion of equine meat have been considered unimportant. However, in a case study by Pomares et al. (2011), three different cases of toxoplasmosis in humans were described where the patients likely had been infected after ingestion of raw horse meat from Brazil and Canada. This would impose a risk of infection in humans both by export of meat from Brazil and also the possibility of ingestion of horsemeat of humans in areas where the economical conditions are poor. More research regarding the seroprevalence of *T. gondii* in horses as well as studies relating seropositivity to presence of tissue cysts in horses would enable a better control of food safety.

Regarding *Neospora* spp., no clinical cases of human neosporosis have been detected. Instead, horses have been shown to be able to develop disease after infection (Finno et al., 2010; Marsh et al., 1996; Wobeser et al., 2009). In Pará and other areas of Brazil, many horse owners lack the funds for veterinary care of their horses. In case of abortions or neurological disease, there is a risk of animal suffering as well as negative impact on the owner's poor economics.

CONCLUSIONS

The present study is the first to present data of prevalence of *Toxoplasma gondii* and *Neospora* spp. in horses in the state of Pará, Brazil. The study found a low frequency of anti-

Toxoplasma gondii as well as anti-*Neospora* spp. antibodies compared to other studies made from other areas of Brazil. IFAT was used for analysis, making it impossible to distinguish between anti-*Neospora caninum* and anti-*Neospora hughesi* antibodies. Antibody titers were generally low for *T. gondii*, but *Neospora* spp. antibody titers were higher than in most other prevalence studies made in Brazil. A statistically significant difference was detected within the determinant groups gender, horse category and municipality for *Neospora* spp. *T. gondii* only had a significant difference between different horse categories. Additional research is necessary to determine whether these differences are real and also to be able to estimate the true prevalence of *T. gondii* and *Neospora* spp. in horses in the state of Pará.

ACKNOWLEDGEMENTS

This degree project was made possible through funding from a Minor Field Study Scholarship from SIDA (Swedish International Development Cooperation Agency) as well as a grant from the Scholarship fund of the Faculty of Veterinary Medicine at Swedish University of Agricultural Sciences in Uppsala.

A special thanks to my Swedish supervisor professor Ulf Emanuelson at Swedish University of Agricultural Sciences for excellent guidance, support and for never leaving an e-mail unanswered for more than a minute. This project could not have been done without you. I also want to thank my Brazilian supervisor professor Antonio Humberto Hhamad Minervino at Universidade Federal do Oeste do Pará for letting me be a part of his study, for all his invaluable help during the field work and for his hospitality.

I want to express my sincere gratitude to Hilda Pena for excellent guidance in the parasitology laboratory at the University of São Paulo, as well as all the persons not mentioned by name at the Universidade Federal do Oeste do Pará and at the Parasitic Diseases Laboratory of the Faculty of Veterinary Medicine and Zootechny of the University of São Paulo who in any way enthusiastically aided me in my work.

Thanks also to all the horse owners and horses in Pará state for their co-operation. Albeit the former more so than the latter.

Last but most importantly, thanks to my partner in crime Emelie Andersson for making both the amazing and the not so amazing moments into memories that will stay with me long after I re-learn to use “I” instead of “we”.

REFERENCES

- Abd-Elnaeim, M.M., Leiser, R., Wilsher & S., Allen, W.R. (2006). Structural and haemovascular aspects of placental growth throughout gestation in young and aged mares. *Placenta*. Vol. 27, pp. 1103–1113.
- Antonello, A.M., Pivoto, F.L., Camillo, G., Braunig, P., Sangioni, L.A., Pompermayer, E. & Vogel, F.S.F. (2012). The importance of vertical transmission of *Neospora* sp. in naturally infected horses. *Veterinary Parasitology*. Vol. 187, pp. 367-370.
- Barber, J.S. & Trees, A.J. (1996). Clinical aspects of 27 cases of neosporosis in dogs. *Veterinary record*. Vol. 139, pp. 439-444.
- Barber, J.S. & Trees, A.J. (1997). Naturally occurring vertical transmission of *Neospora caninum* in dogs. *International Journal for Parasitology*. Vol. 28, pp. 57-64.
- Beghin, N. (2008) Notes on Inequality and Poverty in Brazil: Current situation and challenges. From Poverty to Power. Available on the internet:
http://www.oxfam.org.uk/resources/downloads/FP2P/FP2P_Brazil_Inequality_Poverty_BP_ENG_LISH.pdf [2012-02-05]
- Björkman, C. & Uggla, A. (1999). Serologic diagnosis of *Neospora caninum* infection. *International Journal for Parasitology*. Vol. 29, pp. 1497-1507.
- Camossi, L.G., Silva, A.V. & Langoni, H. (2010). Inquérito sorológico para toxoplasmose em equinos na região de Botucatu-SP. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*. Vol. 62:2, pp. 484-488.
- de Moura, A.B., da Silva, M.O., Farias, J.A., Vieira-Neto, A., de Souza, A.P., Sartor, A.A., Fonteqe, J.H. & Bunn, S. (2013). *Neospora* spp. antibodies in horses from two geographical regions of the state of Santa Catarina, Brazil. *Revista Brasileira de Parasitologia Veterinária*. Vol. 22:4, pp. 597-601.
- de Oliveira, E., de Albuquerque, P.P.F., de Souza Neto, O.L., Faria, E.B., Júnior, J.W.P. & Mota, R.A. (2013). Occurrence of Antibodies to *Toxoplasma gondii* in Mules and Donkeys in the Northeast of Brazil. *Journal of Parasitology*. Vol. 99:2, pp. 343-345.
- Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J. & Uggla, A. (1988). Newly recognized fatal protozoan disease of dogs. *Journal of American Veterinary Medicine Association*, vol. 192, pp. 1269–1285.
- Dubey, J.P. & Porterfield, M.L. (1990). *Neospora caninum* (Apicomplexa) in an aborted equine fetus. *The Journal of Parasitology*. Vol. 76, pp. 732-734.
- Dubey, J.P. & Lindsay, D.S. (1996). A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology*. Vol. 67, pp. 1-59.
- Dubey, J.P., Mattix, M.E. & Lipscomb, T.P. (1996). Lesions of neonatally induced toxoplasmosis in cat. *Veterinary pathology*. Vol. 33, pp. 290-295.
- Dubey, J.P., Lindsay, D.S. & Speer, C.A. (1998). Structures of *Toxoplasma gondii* tachyzoites, bradyzoites and sporozoites and biology and development of tissue cysts. *Clinical Microbiology Reviews*, pp. 267-299.
- Dubey, J.P., Kerber, C.E. & Granstrom, D.E., 1999. Serologic prevalence of *Sarcocystis neurona*, *Toxoplasma gondii*, and *Neospora caninum* in horses in Brazil. *Journal of American Veterinary Medicine Association*. Vol. 215, pp. 970–972.

- Dubey, J.P. & Schares, G. (2006). Diagnosis of bovine neosporosis. *Veterinary parasitology*. Vol. 140, pp. 1-34.
- Dubey, J.P. & Schares, G. & Ortega-Mora, L.M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews*. Vol. 20:2, pp. 323-367.
- Dubey, J.P. (2009). *Toxoplasmosis in animals and humans*. 2. ed. Florida; CRC Press.
- Dubey, J.P., Jenkins, M.C., Rajendran, C., Miska, K., Ferreira, L.R., Martins, J., Kwok, O.C.H. & Choudhary, S. (2011). Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*. *Veterinary Parasitology*. Vol. 181, pp. 382–387.
- Dubey, J.P. & Schares, G. (2011). Neosporosis in animals – the last five years. *Veterinary Parasitology*. Vol. 180, pp. 90-108.
- Encyclopaedia Britannica. (2012). Pará. Available on the internet:
<http://www.britannica.com/EBchecked/topic/442305/Para> [2012-02-05]
- Evers, F., Garcia, J.L., Navarro, I.T., Zulpo, D.L., de Souza Lima Nino, B., de Carvalho Ewald, M.P., Pagliari, S., de Almeida, J.C. & Freire, R.L. (2013). Diagnosis and isolation of *Toxoplasma gondii* in horses from Brazilian slaughterhouses. *Revista Brasileira de Parasitologia Veterinária*. Vol. 22:1, pp. 58-63.
- Fagge, N. (2011) [Electronic] Mail online, december 26. Available on the internet:
<http://www.dailymail.co.uk/news/article-2078596/Brazil-overtakes-UK-sixth-biggest-economy-Britain-falls-South-American-nation-time.html> [2012-02-05]
- Finger, M.A., Villalobos, E.M.C., Lara, M do C.M., Cunha, E.M.S., de Barros Filho, I.R., Deconto, I., Dornbusch, P.T., Ullman, L.S. & Biondo, A.W. (2013). Detection of anti-*Toxoplasma gondii* antibodies in carthorses in the metropolitan region of Curitiba, Paraná, Brazil. *Revista Brasileira de Parasitologia Veterinária*. Vol. 22:1, pp. 179-181.
- Finno, C.J., Eaton, J.S., Aleman, M. & Hollingsworth, S.R. (2010). Equine protozoal myeloencephalitis due to *Neospora hughesi* and equine motor neuron disease in a mule. *Veterinary Ophthalmology*. Vol 13, pp. 259-265.
- Garcia, J.L., Navarro, I.T., Ogawa, L. & Oliveira, R.C. (1999). Seroprevalence of *Toxoplasma gondii* in swine, bovine, ovine and equine, and their correlation with human, felines and canines, from farms in North Region of Paraná State, Brazil. *Ciência Rural*. Vol. 29:1, pp. 91-97.
- Gondim, L.F., McAllister, M.M., Pitt, W.C. & Zemlicka, D.E. (2004). Coyotes (*Canis Latrans*) are definitive hosts of *Neospora caninum*. *International journal of for Parasitology*. Vol. 4, pp. 159-161.
- Gondim, L.F.P., Lindsay, D.S. & McAllister, M.M. (2009). Canine and bovine *Neospora caninum* control sera examined for cross-reactivity using *Neospora caninum* and *Neospora hughesi* Indirect Fluorescent Antibody Tests. *Journal of Parasitology*. Vol. 95:1, pp. 86-88.
- Hoane, J.S., Gennari, S.M., Dubey, J.P., Ribeiro, M.G., Borges, A.S., Yai, L.E., Aguiar, D.M., Cavalcante, G.T., Bonesi, G.L. & Howe, D.K. (2006). Prevalence of *Sarcocystis neurona* and *Neospora* spp. infection in horses from Brazil based on presence of serum antibodies to parasite surface antigen. *Veterinary Parasitology*. Vol. 136, pp. 155–159.
- International Fund for Agricultural Development, IFAD (2011) Enabling poor rural people overcome poverty in Brazil. Available on the internet:
http://www.ifad.org/operations/projects/regions/PL/factsheet/brazil_e.pdf [2012-02-07]

- King, J.S., Slapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T. & Windsor, P.A. (2010). Australian dingoes are definitive host of *Neospora caninum*. *International Journal of Parasitology*. Vol. 40, pp. 945–950.
- Kligler, E.B., Shkap, V., Baneth, G., Mildenberg, Z. & Steinman, A. (2007). Seroprevalence of *Neospora* spp. among asymptomatic horses, aborted mares and horses demonstrating neurological signs in Israel. *Veterinary Parasitology*. Vol. 148, pp. 109–113.
- Langoni, H., Silva, A.V., Pezerico, S.B. & Lima, V.Y. (2007). Utilization of modified agglutination test and indirect immunofluorescent antibody test for the detection of *Toxoplasma gondii* antibodies in naturally exposed horses. *Brazilian Journal of Veterinary Research and Animal Science*. Vol. 44, pp. 27–32.
- Lyon, C. (2010). Update on the diagnosis and management of *Neospora caninum* infections in dogs. *Protozoal Diseases in Small Animals*. Vol. 25:3, pp. 170–175.
- Mai, K., Sharman, P.A., Walker, R.A., Katrib, M., Souza, D.D., McConville, M.J., Wallach, M.G., Belli, S.J., Ferguson, D.J. & Smith, N.C. (2009). Oocyst wall formation and composition in coccidian parasites. *Mem. Inst. Oswaldo Cruz*, vol. 104(2), pp. 81–289.
- Marsh, A.E., Barr, B.C., Madigan, J., Lakritz, J., Nordhausen, R. & Conrad, P.A. (1996). Neosporosis as a cause of equine protozoal myeloencephalitis. *Journal of Veterinary Medicine Association*. Vol. 209 (11), pp. 1907–1913.
- Marsh, A.E., Barr, B.C., Packham, A.E. & Conrad, P.A. (1998). Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). *Journal of Parasitology*, vol. 84, pp. 983–991.
- McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A. & McGuire, A.M. (1998). Dogs are definitive hosts of *Neospora caninum*. *International Journal of Parasitology*. Vol. 28, pp. 1473–1478.
- McAllister, M.M. (2005). A decade of discoveries in veterinary protozoology changes our concept of “subclinical” toxoplasmosis. *Veterinary Parasitology*. Vol. 132, pp. 241–247.
- McCann, C.M., Vyse, A.J., Salmon, R.L., Thomas, D., Williams, D.J.L., McGarry, J.W., Pebody, R. & Trees, A.J. (2008). Lack of serologic evidence of *Neospora caninum* in humans, England. *Emerging Infectious Diseases*. Vol. 14, pp. 978–980.
- McDole, M.G. & Gay, J.M. (2002). Seroprevalence of antibodies against *Neospora caninum* in diagnostic equine serum samples and their possible association with fetal loss. *Veterinary Parasitology*. Vol. 105, pp. 257–260.
- Pitel, P-H., Romand, S., Pronost, S., Foucher, N., Gargala, G., Maillard, K., Thulliez, P., Collobert-Laugier, C., Tainturier, D., Fortier, G. & Ballet, J.J. (2003). Investigation of *Neospora* spp. antibodies in aborted mares from Normandy, France. *Veterinary Parasitology*. Vol. 118, pp. 1–6.
- Pomares, C., Ajzenberg, D., Bornard, L., Bernadin, G., Hasseine, L., Dardé, M. & Marty, P. (2011). Toxoplasmosis and horse meat, France [letter]. *Emerging Infectious Disease* [serial on the Internet] 2011 July. Available at: <http://dx.doi.org/10.3201/eid1707.101642>. Accessed 2014-05-01.
- Powell, C.C., Brewer, M. & Lappin, M.R. (2001). Detection of *Toxoplasma gondii* in the milk of experimentally infected lactating cats. *Veterinary parasitology*. Vol. 102, pp. 29–33.
- Pusterla, N., Conrad, P.A., Packham, A.E., Mapes, S.M., Finno, C.J., Gardner, I.A., Barr, B.C., Ferraro, G.L. & Wilson, W.D. (2011). Endogenous transplacental transmission of *Neospora hughesi* in naturally infected horses. *The Journal of Parasitology*. Vol. 97, pp. 281–285.

- Taylor, M.A., Coop, R.L. & Wall, R.L. (2007). *Veterinary Parasitology*. 3. ed. Oxford; Blackwell publishing.
- Tenter, A.M., Heckeroth, A.R. & Weiss, L.M. (2000). *Toxoplasma gondii* – from animals to man. *International Journal for Parasitology*. Vol. 30, pp. 1217-1258.
- Toscan, G., Vogel, F.S.F., Cadore, G.C., Cezar, A.S., Sangioni, L.A., Pereira, R.C.F., Oliviera, L.S.S. & Lopes, S.T.A. (2011). Occurrence of antibodies ant-*Neospora* spp. in cart horses and Crioula breed horses from Rio Grande do Sul State. *Arquivos da Escola Veterinária da Universidade Federal de Minas Gerais*. Vol. 63:1, pp. 258-261.
- Trees, A.J., Guy, F., Tennant, B.J., Balfour, A.H., & Dubey, J.P. (1993). Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. *Veterinary Record*. Vol. 132, pp. 125-126.
- Turner, C.B. & Savva, D. (1990). Evidence of *T. gondii* in an equine placenta. *Veterinary Record*. Vol. 127, pp. 96-96.
- Turner, C.B. & Savva, D. (1991). Detection of *T. gondii* in equine eyes. *Veterinary Record*. Vol. 129, pp. 128-128.
- Turner, C.B. & Savva, D. (1992). Transplacental infection of a foal with *T. gondii*. *Veterinary Record*. Vol. 131, pp. 179-180.
- UNESCO. (2011) UNESCO office in Brasilia, Social and Human Sciences, Poverty reduction. Available on the internet: <http://www.unesco.org/new/en/brasil/social-and-human-sciences/poverty-reduction/> [2012-02-06]
- Veronesi, F., Diaferia, M., Mandara, M.T., Marenzoni, M.L., Cittadini, F. & Piergili Fioretti, D. (2008). *Neospora* spp. infection associated with equine abortion and/or stillbirth rate. *Veterinary Research Communications*. Vol. 32, pp. 223-226.
- Villalobos, E.M., Ueno, T.E., de Souza, S.L., Cunha, E.M., do Carmo Custodio de Souza Hunold Lara, M., Gennari, S.M. & Soares, R.M. (2006). Association between the presence of serum antibodies against *Neospora* spp. and fetal loss in equines. *Veterinary Parasitology*. Vol. 142, pp. 372–375.
- Villalobos, E.M., Furman, K.E., Lara M. do C.M., Cunha, E.M., Finger, M.A., Busch, A.P., de Barros Filho, I.R., Deconto, I., Dornbusch, P.T. & Biondo, A. W. (2011). Detection of *Neospora* sp. antibodies in cart horses from urban areas of Curitiba, Southern Brazil. *Revista Brasileira de Parasitologia Veterinária*. Vol. 21:1, pp. 68-70.
- Williams, D.J.L., Hartley, C.S., Björkman, C. & Trees, A.J. (2009). Endogenous and exogenous transplacental transmission of *Neospora caninum* – how the route of transmission impacts on epidemiology and control of disease. *Veterinary Parasitology*. Vol. 136, pp. 1895–1900.
- Wobeser, B.K., Godson, D.L., Rejmanek, D. & Dowling, P. (2009). Equine protozoal myeloencephalitis caused by *Neospora hughesi* in an adult horse in Saskatchewan. *Canadian Veterinary Journal*. Vol. 50, pp. 851-853.

Oral sources

- Prof. Antonio Humberto Hamad Minervino, Federal University of Western Pará (UFOPA). Interview during fieldwork in Santarém, state of Pará, Brazil. September 2012.