SOLANGE DE OLIVEIRA

Experimental oral infection in chickens (*Gallus gallus domesticus*) with *Neospora caninum* (NC-SP1) oocysts

São Paulo
2017

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Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

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Ph.D. Hilda Fátima de Jesus Pena

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CERTIFICADO

Certificamos que a proposta intitulada "Infecção experimental oral com oocistos de Neospora caninum, isolado NC-SP1, em galináceos (Gallus gallus domesticus)", protocolada sob o CEUA nº 8634270114, sob a responsabilidade de **Hilda Fátima de Jesus Pena** *e equipe; Solange de Oliveira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 15/10/2014.

We certify that the proposal "Experimental oral infection of chickens (Gallus gallus domesticus) with Neospora caninum (NC-SP1) oocysts", utilizing 6 Bovines (6 males), 6 Dogs (6 males), 288 Birds (288 females), 288 Gerbils (288 males), protocol number CEUA 8634270114, under the responsibility of **Hilda Fátima de Jesus Pena** *and team; Solange de Oliveira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/15/2014.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 02/2014 a 11/2017 Área: Medicina Veterinária Preventiva E Saúde Animal

Origem:	Não aplicável biotério				
Espécie:	Bovinos	sexo: Machos	idade: a	N:	6
Linhagem:	N/A		Peso: a		
Origem:	Animais provenientes de doação espontânea				
Espécie:	Cães	sexo: Machos	idade: a	N:	6
Linhagem:	SRD		Peso: a		
Origem:	Animais provenientes de estabelecimentos comerciais				
Espécie:	Aves	sexo: Fêmeas	idade: a	N:	144
Linhagem:	Poedeiras		Peso: a		
Origem:	Animais provenientes de estabelecimentos comerciais				
Espécie:	Aves	sexo: Fêmeas	idade: a	N:	144
Linhagem:	Poedeiras		Peso: a		
Origem:	Animais provenientes de estabelecimentos comerciais				
Espécie:	Gerbil	sexo: Machos	idade: a	N:	288
Linhagem:	Gerbil		Peso: a		

Resumo: Neospora caninum é um protozoário apicomplexa, heteroxeno e eurixeno, que causa doenças neuromusculares em cães e abortamentos em bovinos. Cães domésticos, coiotes, dingos e lobos cinzentos são considerados hospedeiros definitivos, infectando-se por ingestão de tecidos de hospedeiros intermediários infectados e excretam oocistos não-esporulados que se tornam infectantes no ambiente. Algumas pesquisas mostraram que galinhas estão associadas à maior prevalência de bovinos infectados e abortamentos em rebanhos. Estudos moleculares e sorológicos mostraram que galinhas podem se infectar por N. caninum, no entanto não há experimentos utilizando a inoculação oral com oocistos de N. caninum que, do mesmo modo que ocorre para Toxoplasma gondii, deve ser a via natural de infecção para esses animais.

Local do experimento:

São Paulo, 20 de setembro de 2017

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Claudia Madalena Cabrera Mori
Vice-Presidente
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

EVALUATION FORM

Author: OLIVEIRA, Solange

Title: **Experimental oral infection in chickens (*Gallus gallus domesticus*) with *Neospora caninum* (NC-SP1) oocysts**

Thesis submitted to the Postgraduate Program in Experimental Epidemiology Applied to Zoonoses of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

Prof. _____

Institution:_____ Decision: _____

DEDICATION

To my grandparents, Doraci and Vivaldo, from whom I learned that we should never give up!

ACKNOWLEDGEMENTS

I would like to thank all the people who contributed in some way to the work described in this thesis.

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Hilda FJ Pena for the continuous support of my PhD study and research, for her friendship, motivation, enthusiasm, patience and immense knowledge.

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I owe a very important debt to my friends. You should know that your presence in my life is worth more than I can express on paper. So, thanks for cheering me up when I have rough days. Thanks for encouraging me and listening to me. It is impossible to count how many beers we had drunk together, many thanks for all of them!

My special thanks to people who gave to our dogs a beautiful home!

Last but not the least, I would like to thank Mom and Dad, my sister and my old friends for supporting me spiritually throughout my life!

RESUMO

OLIVEIRA, S. **Infecção experimental oral de galináceos (*Gallus gallus domesticus*) com oocistos de *Neospora caninum* (NC-SP1).** [**Experimental oral infection in chickens (*Gallus gallus domesticus*) with *Neospora caninum* (NC-SP1) oocysts**]. 2017. 70 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Neospora caninum é um parasita formador de cistos teciduais que pertence ao filo Apicomplexa. O cão doméstico e outros canídeos são os hospedeiros definitivos de *N. caninum*, pois podem excretar nas fezes os oocistos, o estágio ambientalmente resistente do parasita. Parasitas viáveis foram isolados de diversas espécies, principalmente herbívoros, confirmando seu papel como hospedeiros intermediários. A importância das aves no ciclo biológico de *N. caninum* ainda não está bem definida. Vários experimentos, utilizando taquizoítas como inóculo, foram realizados em aves domésticas e silvestres e os resultados não foram conclusivos. Na natureza, a via de transmissão mais provável para as galinhas é a ingestão de oocistos. Este trabalho teve como objetivo avaliar a infecção por *N. caninum* em galináceos infectados por via oral com oocistos de um novo isolado de referência de *N. caninum*. Inicialmente, 400g de cérebro de um bovino adulto naturalmente infectado, apresentando anticorpos anti-*N. caninum* por meio da reação de imunofluorescência indireta - RIFI (título = 200), foram oferecidos para um cão de 2 meses de idade. Oocistos *Neospora*-like foram visualizados 7 dias pós inoculação (p.i.). A sequência final obtida a partir do DNA extraído dos oocistos, baseada no marcador ITS-1, teve 99% de similaridade com sequências homólogas de *N. caninum*. Gerbilos (*Meriones unguiculatus*) foram inoculados com diferentes doses de oocistos (10, 100 e 1000 oocistos) por via oral, e todos eles permaneceram clinicamente normais e anticorpos contra *N. caninum* foram detectados 14 dias p.i. por meio da RIFI (título ≥ 50). O homogenado do cérebro de um gerbilo infectado foi inoculado em uma monocamada de células da linhagem celular Vero e taquizoítas foram observados no cultivo celular 24 dias p.i.. A genotipagem por microssatélites do DNA extraído desses taquizoítas revelou um perfil genético único ao novo isolado de referência, designado NC-SP1. Trinta galináceos (*Gallus gallus domesticus*) da linhagem White Leghorn foram então inoculados via papo aos 21 dias de idade com oocistos de NC-SP1 (200 oocistos por ave). As

eutanásias foram realizadas em intervalos de 7 dias para cada grupo de três aves, durante 9 semanas, e um grupo foi desafiado com a mesma dose de oocistos aos 37 dias p.i. e acompanhado por 11 semanas. Amostras de sangue foram colhidas semanalmente, e os soros testados por meio da RIFI. Os tecidos das aves foram analisados por meio da PCR, PCR quantitativa (qPCR) e imunohistoquímica (IHQ). Dois grupos de aves foram eutanasiados aos 138 e 159 dias p.i. e os tecidos foram oferecidos para dois cães sem raça definida com aproximadamente 45 dias de idade. Os galináceos não soroconverteram (título < 5), e o DNA (PCR e qPCR) e o antígeno (IHQ) de *N. caninum* não foram detectados nos tecidos das aves. Oocistos de *N. caninum* não foram excretados pelos cães. Nestas condições experimentais, os galináceos foram resistentes à infecção por *N. caninum*.

Palavras-chave: *Neospora caninum*. Isolamento. Oocistos. Infecção experimental. Galináceos.

ABSTRACT

OLIVEIRA, S. **Experimental oral infection in chickens (*Gallus gallus domesticus*) with *Neospora caninum* (NC-SP1) oocysts.** [**Infecção experimental oral de galináceos (*Gallus gallus domesticus*) com oocistos de *Neospora caninum* (NC-SP1)**]. 2017. 70 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2017.

Neospora caninum is a tissue-cyst forming parasite that belongs to the phylum Apicomplexa. Dogs and other canids are the definitive hosts of *N. caninum* and they are responsible to excrete oocysts, the environmentally resistant stage of the parasite. Viable parasites have been isolated from a variety of species, especially herbivorous, confirming their role as intermediate hosts. The importance of birds in the life cycle of *N. caninum* is not clear. Several experiments, using tachyzoites as inoculum, were conducted in domestic and wild birds and the results were not conclusive. In nature, the possible mode of transmission in chickens is the ingestion of oocysts. This work aimed to evaluate the infection by *N. caninum* in chickens orally inoculated with oocysts obtained from a new isolate of *N. caninum*. For that, approximately 400 g of brain from a naturally infected adult cattle, showing anti-*N. caninum* antibodies by means of the immunfluorescent antibody test - IFAT (titre = 200), were fed to a 2-month-old dog. *Neospora*-like oocysts were observed on day 7 post-inoculation (p.i.). The DNA obtained from oocysts was molecularly characterized using the ITS-1 marker and the final sequence was 99% identical to homologous sequences of *N. caninum.* Gerbils (*Meriones unguiculatus*) were orally inoculated with different doses of oocysts (10, 100 and 1000 oocysts), and all of them remained clinically normal and developed *N. caninum* antibodies 14 days p.i. (titre ≥ 50 by IFAT). Brain homogenate from an infected gerbil was seeded into a monolayer of Vero cells and tachyzoites were visualized at 24 days p.i.. Microsatellite genotyping using DNA from the tachyzoites revealed a unique genetic profile for this new reference isolate, named NC-SP1. Thirty White Leghorn chickens (*Gallus gallus domesticus*) were orally inoculated with viable *N. caninum* oocysts from the NC-SP1 isolate (200 oocysts per bird) via the crop at 21 days of age. Groups of three birds were euthanized at intervals of 7 days during 9 weeks; one group was challenged with the same oocysts dose at 37 days p.i. and observed for 11 weeks. Blood samples were collected weekly, and sera were tested

by IFAT. Chicken tissues were analyzed using PCR, quantitative PCR and immunohistochemistry (IHC). Two mongrel dogs, approximately 45 days of age were fed with tissues from chickens euthanized at 138 and 159 days p.i.. The chickens did not seroconvert (titre < 5), and neither DNA (PCR and qPCR) nor antigen (IHC) of *N. caninum* was detected in inoculated chicken tissues. In addition, no oocyst excretion by the dogs was observed. In these experimental conditions, the chickens were resistant to *N. caninum* infection.

Keywords: *Neospora caninum*. Isolation. Oocysts. Experimental infection. Chickens.

SUMMARY

1. INTRODUCTION

Neospora caninum is a tissue-cyst forming parasite that belongs to the phylum Apicomplexa. Since the first recognition in dogs in 1984 in Norway (Bjerkas et al., 1984) and description of the new genus and species by Dubey et al. (1988), *N. caninum* has been associated with neuromuscular disorders in dogs and has been identified as an important cause of abortion and neonatal mortality in cattle worldwide (Dubey et al., 2017).

Dogs (*Canis lupus familiaris*) were the first definitive host described for *N. caninum*. McAllister et al. (1998) fed dogs with tissue cysts in infected mouse carcasses, and the dogs shed oocysts that were morphologically similar to *Toxoplasma gondii*, *Hammondia hammondi* and *Hammondia heydorni*. In addition, a year after, Lindsay et al. (1999) confirmed this discovery. Later on, related canids such as coyotes (*Canis latrans*) (Gondim et al., 2004), dingoes (*Canis lupus dingo*) (King et al., 2010) and grey wolves (*Canis lupus lupus*) (Dubey et al., 2011) were also recognized as definitive hosts. Viable parasites have been isolated from a variety of mammals, especially herbivores, confirming they act as intermediate hosts.

The life cycle of *N. caninum* involves three known infectious stages: tachyzoites, bradyzoites and sporozoites within sporulated oocysts. Tachyzoites and bradyzoites, fast-replicating and encysted stages of the parasite, respectively, are the stages found in intermediate hosts (Dubey et al., 2002). The oocysts are the environmentally resistant stage of the parasite (Uzeda et al., 2007; Neto et al., 2011).

All three infectious stages of *N. caninum* are involved in the transmission of the parasite. The definitive host sheds unsporulated oocysts that in as few as 48h outside the host become sporulated and infectious; after sporulation, the oocysts contain two sporocysts, each of which contains four sporozoites (Lindsay et al., 1999; Dubey et al., 2006; Reichel et al., 2007). When these oocysts are ingested by an intermediate host through food or contaminated water (horizontal transmission), the sporozoites are released and transform into tachyzoites, which are able to proliferate and disseminate to different organs (Dubey and Lindsay 1996; Hemphill et al., 2006). Eventually, under host immune pressure, the tachyzoites transform into bradyzoites, which develop within the tissue cyst and can persist for several months (Dubey and Lindsay 1996; Hemphill et al., 2006). Carnivores probably become infected horizontally by ingesting

tissues containing bradyzoites (Dubey and Schares 2011). Although fecal transmission of the parasite appears to be less important in dogs and the results need confirmation (Bandini et al., 2011).

Neospora caninum is one of the most efficiently transplacentally transmitted parasites in cattle (Dubey et al., 2007). Historically, vertical transmission of *N. caninum* was first recognized in dogs but, unlike in cattle, this transmission is considered highly variable and not likely to persist in nature in the absence of horizontal transmission of infection (Barber and Trees 1998; Dubey et al., 2005; Reichel et al., 2007).

The vertical transmission occurs when tachyzoites are transmitted from an infected dam to her fetus, via placenta, and can be classified depending on the origin of the infection as endogenous or exogenous (Trees and Williams 2005; Williams et al., 2009). Endogenous transplacental infection is defined as a fetal infection from a recrudescent maternal infection acquired before pregnancy, maintaining infection within the herd. In contrast, exogenous transplacental infection occurs because of an infection of the dam during pregnancy and is associated with an epidemic pattern of abortion (Trees and Williams 2005; McCann et al., 2007; Williams et al., 2009). Vertical transmission was observed in other species such as horse, deer, goat, water buffalo, and sheep (Lindsay et al., 1996; Dubey et al., 2013; Mesquita et al., 2013; Chryssafidis et al., 2015; Filho et al., 2017).

Oocysts are the key in the epidemiology of neosporosis and they are morphologically similar to oocysts of *T. gondii* and *H. hammondi* in cat feces, and to *H. heydorni* in dog feces (Dubey et al., 2002). However, microscopic examinations are not enough to differentiate *N. caninum* oocysts in dog feces; therefore, a molecular approach should be applied (Hill et al., 2001; Šlapeta et al., 2002; Monteiro et al., 2007).

Several serological techniques have been developed to search for *N. caninum* antibodies, especially to represent the serological status in cattle. Serological techniques including ELISAs (enzyme-linked immunosorbent assays), IFAT (indirect fluorescence antibody test), NAT (*Neospora* agglutination test), LAT (latex agglutination test), IB (Immunoblotting), and RIT (rapid immunochromatographic test) have been used with different purposes for serological diagnosis of *N. caninum*, but there is no true gold standard assay (reviewed by Guido et al., 2016). Because IFAT was the first serological test used to detect anti-*N. caninum* antibodies, it is still the

reference method for serodiagnosis of *N. caninum* infection (Dubey et al., 1988; Schares et al., 1998).

In serological tests, cross-reactivity can occur among closely related parasites; in this sense, infections caused by *N. caninum* and *Neospora hughesi* cannot be serologically discriminated (Nishikawa et al., 2002; Gondim et al., 2017). On the other hand, the closely related parasite *H. heydorni* is known for not cross-reacting with *N. caninum*, however, further studies using larger numbers of sera from *H. heydorni* infected animals are necessary to confirm the absence of this cross-reactivity (Gondim et al., 2017). In addition, it is important to emphasize that the description of a new intermediate host based only on the detection of antibodies should be made with caution.

Isolation of viable parasite is a very important tool for biological and molecular studies of *N. caninum*; nevertheless, it is not an easy task and not all isolates could be cultivated in cell culture and cryopreserved for future studies (Vianna et al., 2005; Dubey and Schares, 2011). The NC1 strain, used in most of the experimental studies, was the first isolate of *N. caninum* and it has been passaged in animal models and cell culture several times (Dubey et al., 1988). After that, *N. caninum* isolates from clinical cases or asymptomatic animals have been described and differences in pathogenicity amongst these isolates have been reported (Regidor-Cerrillo et al., 2008; Rojo-Monteiro et al, 2009; Dellarupe et al., 2014).

Isolates of *N. caninum* from different hosts can have their own molecular signature as determined by multilocus microsatellite analysis. These genetic markers have been shown to be the most suitable polymorphic markers for the typing of *N. caninum* isolates, and have been applied to genetically characterize sets of isolates (Regidor-Cerrillo et al., 2006; 2013; Pedraza-Díaz et al., 2009; Brom et al., 2014). However, knowledge about the genetic diversity of *N. caninum* is limited. The main reason is that there are very few viable isolates available worldwide. In Brazil, only three *N. caninum* isolates were well described: NC-Bahia strain was isolated from a naturally infected dog with clinical signs (Gondim et al., 2001), Nc-Goiás (García-Melo et al., 2009) and NC-SP1 (Oliveira et al., 2017) were isolated from asymptomatic cattle and display variation in three or four out of nine markers used in microsatellite genotyping (Oliveira et al., 2017).

Historically, birds were inoculated to be evaluated as potential definitive host for *N. caninum*. Baker et al. (1995) fed infected rodent tissues to four species of

carnivorous birds (*Buteo jamaicensis*, *Cathartes aura*, *Tyto alba* and *Corvus brachyrhynchus*) but none of the birds shed oocysts nor seroconvert. After that, several studies were conducted to clarify if birds may be involved in the life cycle of *N. caninum*.

Some studies point out that domestic birds are a risk factor for the transmission of *N. caninum*. Bartles et al. (1999) and Ould-Amrouche et al. (1999) showed that the number of *N. caninum* infected animals and the risk of abortion outbreaks in dairy cattle were associated with the presence of dogs, cats, rabbits, chickens, ducks, and geese.

Experimentally, species such as *Columba livia* (domestic pigeons) and *Poephila guttata* (zebra finches) were inoculated with *N. caninum* tachyzoites, and then observed during six weeks post-inoculation (p.i.). *N. caninum* infection was confirmed in *C. livia* through antibody detection (IFAT) and presence of DNA in brains. The parasites were re-isolated in cell culture; however, *P. guttata* was negative in all analyses (McGuire et al., 1999). Similar results were observed by Mineo et al. (2009), in which one out of four inoculated domestic pigeons died at 25 days p.i. and *N. caninum* was found in different tissues using immunohistochemistry (IHC). In another study, Oliveira et al. (2013) used tachyzoites of *N. caninum* to infect quails (*Coturnix cotunirx japonica*); and the seroconversion was observed in the first two weeks p.i. (7^{th} and 14^{th} days p.i.). Three dogs were fed quail tissues 60 days p.i. and did not shed oocysts, demonstrating that quails are resistant to infection with *N. caninum*.

Domestic chickens were also used to comprehend the infection dynamics of *N. caninum* in this species. Furuta et al. (2007) infected 7-day-old chicks and laying hens intraperitoneally with tachyzoites of *N. caninum* (NC1). Chickens had an asymptomatic infection, with seroconversion at 15 days p.i. and systemic replication of the parasite detectable by IHC; however, at the end of the experiment (60 days p.i.) all chickens were seronegative (IFAT) with the absence of parasites in tissues (IHC). On the other hand, Munhoz et al. (2014) affirmed that chickens are resistant to infection with *N. caninum* tachyzoites. In this experiment, 90-day-old chickens were subcutaneously inoculated with tachyzoites, and observed during 70 days p.i.; at this point, tissue samples were collected for histopathology, IHC and PCR, moreover, brain tissue and pectoral muscle were fed to two dogs; no changes consistent with infection were detected and no dog excreted oocysts.

Neospora caninum DNA has been detected in different species of domestic and wild birds. Costa et al. (2008) tested brains from seropositive chickens and detected *N. caninum* DNA in six out of 10 chickens, whereas Romero et al. (2016) found two

positive brains out of 50 (4%) sampled chickens. DNA of *N. caninum* has been found in species such as *Passer domesticus* (sparrow), *Pica pica* (magpie), *Buteo buteo* (common buzzard), and species from the genus *Anas* and *Vanellus vanellus* (waterfowls) (Gondim et al., 2010; Darwich et al., 2012; Rocchigiani et al., 2017). Only one study described *N. caninum* isolation attempts from seropositive chickens, for that, interferon-gamma knockout mice were bioassayed with chicken tissues, and the isolation failed (Gonçalves et al., 2012).

Anti-*N. caninum* antibodies are frequently reported in free-range chickens and wild birds worldwide. Martins et al. (2011) reported a seroprevalence of 39.5% (524/1324, IFAT) in chickens from different countries of the Americas, whereas Costa et al. (2008) reported 23.5% (47/200, IFAT) seropositive chickens in the state of Bahia, Brazil, and Sayari et al. (2016) found 17.33% (26/150, MAT) in chickens from Iran. For wild birds, Mineo et al. (2011) tested sera from 294 captive and free-range birds from 17 species and nine orders from Brazil, and anti-*N. caninum* antibodies were not detected (IFAT); however, Molina-López et al. (2012) reported 35.8% (24/67, IFAT) of seropositivity in common ravens (*Corvus corax*) in the Northeast of Spain, and Barros et al. (2017) found 31.72% (79/249, ELISA) of seropositive eared doves (*Zenaida auriculata*) for *N. caninum* in Southern Brazil.

The main purpose of the present study was to experimentally infect chickens with oocysts of *N. caninum*, because the importance of birds in the life cycle of *N. caninum* is yet not clear. In nature, the possible mode of infection in chickens is the ingestion of oocysts; however, in all experimental infection of birds, only tachyzoites were used as inoculum. In addition, chickens were recognized as intermediate host of *N. caninum* based on molecular findings; nonetheless, viable parasites have never been isolated from naturally infected chickens. To achieve our main purpose, first we isolated *N. caninum* from an asymptomatic bovine and described the biological and molecular features of the new isolate, named NC-SP1, and then proceeded to the experimental infection in chickens using oocysts of NC-SP1.

The second chapter reports the isolation, biological and molecular characterization of a new isolate of *N. caninum*, named NC-SP1. The third chapter describes the experimental infection of chickens using oocysts of *N. caninum* (NC-SP1). It is important to highlight that the experimental infection of chickens with tachyzoites did not belong to the original purpose of this thesis, it was a collaboration with Dr. JP Dubey and his team, from USDA (United States Department of Agriculture,

Beltsville, Maryland), during the publication process. The formatting of chapters two and three follows the journal format, in which the papers were published, and the content of the chapters is identical to that of the papers available on the journal's website and the references are located in the footnotes.

2. ISOLATION AND BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Neospora caninum* (NC-SP1) FROM A NATURALLY INFECTED ADULT ASYMPTOMATIC CATTLE (*Bos taurus*) IN THE STATE OF SÃO PAULO, BRAZIL

ABSTRACT

The biological and genetic diversity of *Neospora caninum* is very limited because of availability of only a few viable isolates worldwide. This study describes the isolation and biological and molecular characterization of a new viable isolate of *N. caninum* (NC-SP1), from a cattle in Brazil. Approximately 400 g of brain from a naturally infected adult male cattle from an abattoir was fed to a 2-month-old dog. *Neospora*-like oocysts were observed on day 7 post-inoculation (PI) and the duration of oocyst shedding was 14 days. The DNA obtained from oocysts was characterized molecularly and the final sequence was 99% identical to homologous sequences of *N. caninum* available in GenBank®. For bioassay, gerbils (*Meriones unguiculatus*) were orally inoculated with 10, 100 and 1000 oocysts; all gerbils remained clinically normal but developed *N. caninum* antibodies 14 days PI. Cell culture isolation was successful using the brain homogenate from one of the gerbils and tachyzoites were observed 24 days PI. Microsatellite genotyping revealed a unique genetic profile for this new reference isolate.

Key words: Neosporosis. Cattle. Bioassay. Oocyst shedding. Gerbils. Microsatellite analysis.

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2.1 INTRODUCTION

Neospora caninum is an important cause of abortion in cattle worldwide. It has a wide host range and can cause clinical disease in several species of animals, including dogs (Dubey *et al.* 2017). It is morphologically and ancestorily related to *Toxoplasma gondii*, but canids (dogs, coyote, dingo and wolf) and not felids are its definitive hosts.

Unlike *T. gondii*, *N. caninum* is very difficult to isolate from naturally infected animals. For this reason, little is known of the genetic variability among *N. caninum* isolates from different hosts and most of the isolates were obtained from clinical cases. This limitation is apparent when a comparison is made: for *T. gondii* 1457 isolates (Shwab *et al.* 2014) compared with 108 *N. caninum* isolates (Regidor-Cerrillo *et al.* 2013) were analysed.

The aims of this study were to isolate viable *N. caninum* from cattle, to perform biological and molecular characterization and to provide a new *N. caninum* isolate that may be used as a reference for further studies.

2.2 MATERIALS AND METHODS

2.2.1 Naturally infected cattle

Brain and serum samples were collected from nine cattle slaughtered in an abattoir in the municipality of Santa Rita do Passa Quatro, state of São Paulo, Brazil. Serum samples were tested by indirect fluorescent antibody test (IFAT) to detect antibodies against *N. caninum* using cut-off of 1:100. Tachyzoite cell cultures, derived from the NC-1 strain of *N. caninum* was used as antigen (Dubey *et al*. 1988). Secondary fluorescein isothiocyanate (FITC)-labelled conjugated IgG anti-bovine

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antibodies were used (Sigma-Aldrich®, Saint Louis, MO, USA). One adult, male cattle had an IFAT titre of 200 for *N. caninum* antibodies and was used for the present study.

Approximately 400 g of brain from the seropositive cattle was cut into small pieces and pooled. Around 10 g was separated for trypsin digestion and four aliquots were collected for further PCR analysis. The remainder was used to isolate *N. caninum* by bioasssay in a dog.

2.2.2 Bioassay in a dog

The brain tissue was mixed with dry commercial dog food and fed over 3-days to a 2-month-old female mixed-breed *N. caninum* seronegative dog (IFAT titre <50). The dog was donated by the owner to the experiment. This dog had never eaten raw meat and was kept on a diet of dry commercial dog food during the experimental period. Blood samples from the dog were collected weekly via the cephalic vein [0 - 135 days post-inoculation, (PI)] and IFAT tests were performed using FITC-labelled conjugated IgG anti-dog antibodies (KPL® Inc., Gaithersburg, MD, USA) with a cut-off point of 1:50.

Fecal samples were examined daily (0-40 days PI) for detection of *Neospora*-like oocysts by means of a standard sucrose flotation technique. Daily output of oocysts as described (Pena *et al*. 2007). When oocysts were observed, the faecal samples were mixed with 2% H_2SO_4 for sporulation at 25°C and thereafter stored at 4°C.

To measure the size of the oocysts, images of 100 unsporulated oocysts were captured at the magnification of 400X and analysed using the Image-Pro Plus® software, version 5.1 (Media Cybermetics, Inc., Silver Spring, MD, USA).

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2.2.3 Brain inoculation in rodents and cell culture

Around 10 g of the cattle brain tissue were digested with 0.05% trypsin for 1 h at 37°C (Dubey *et al.* 2013). After the digestion, the homogenized suspension was washed three times with HBSS buffer (Hank's Balanced Salt Solution, GIBCO®, Grand Island, NY, USA) by centrifuging it at 1500 x *g* for 10 min and the resultant pellet was suspended in RPMI 1640 medium (Roswell Park Memorial Institute medium). Two flasks containing a monolayer of VERO cells were seeded with the sediment obtained (500 µL per flask). Two gerbils (*Meriones unguiculatus*) were intraperitoneally inoculated (500 µL per animal).

2.2.4 Bioassay of oocysts in gerbils and isolation in cell culture

Eight groups of two gerbils each were orally inoculated with 200 oocysts per gerbil. Every week, blood samples were collected from all the animals for serological analysis and one group was randomly euthanized. Tissues were collected from each animal for PCR assay. For isolation in cell culture, nine gerbils, divided into three groups, were orally infected with 10^1, 10^2 and 10^3 oocysts. The brains of the gerbils were aseptically collected, homogenized, digested with 0.05% trypsin or acid-pepsin solution (pepsin, 1.3 g; NaCl, 2.5 g; HCl, 3.5 mL; and distilled water, 250 mL) as described in (Dubey 1998), and inoculated into a monolayer of VERO cells.

The serum samples were tested by means of IFAT using anti-gerbil IgG FITC-labelled conjugate (Immunology Consultants Laboratory® Inc., Portland, OR, USA). The cut-off was 1:50; the positive samples were diluted until the endpoint and the titres were then attributed.

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2.2.5 DNA extraction and PCR assay

DNA from animal tissues was obtained using DNeasy Blood and Tissue Kit® (Qiagen Inc., Hilden, Germany), in accordance with the manufacturer's instructions.

To extract DNA from the oocysts, the oocysts were ruptured by means of six cycles of freezing in liquid nitrogen (-192°C for 2 min) and thawing at 37°C. After this pre-treatment, DNA extraction was done using the QIAamp DNA Stool Mini Kit® (Qiagen).

The PCR assays were performed using a pair of primers based on the Nc-5 gene (Np6 plus and Np21 plus) (Müller *et al*. 1996) for both the animal tissues and oocysts; and based on the ITS-1 region [(JS4) (Šlapeta *et al*. 2002) and (CT2c, JS4b and CT2b) (Soares *et al*. 2011)] for the oocysts. The amplified DNA was viewed by electrophoresis on 2% agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen™, Carlsbad, CA, USA).

2.2.6 Identity of *Neospora*-like oocysts

The DNA of the *Neospora*-like oocysts were tested with an ITS-1 nested PCR-RFLP, in order to confirm the identity of the *Neospora*-like oocysts and to rule out mixed infection with oocysts of *Hammondia heydorni*. The primers were designed based on 18S and 5.8SrRNA coding genes and flanked the ITS-1 region, as described by Soares *et al*. (2011). The amplicons were digested using the restriction endonuclease TaqαI. Also, the oocysts were tested with Np6 plus and Np21 plus primers. The amplified DNA was viewed as described above.

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2.2.7 ITS-1 sequencing of the isolate NC-SP1

DNA from tachyzoites was obtained from the cell culture. The ITS1-PCR products based on sense and antisense primers (JS4 and CT2b) were sequenced using the BigDye® Terminator v3.1 cycle sequencing kit and the sequencing products were analysed in the ABI 3730 DNA Analyser (Life Technologies; Applied Biosystems, Foster City, CA, USA). The PCR amplicons were sequenced four times in both directions. The sequences were assembled and the contig was formed with the phred base-calling and phrap assembly tools, which are available in the Codoncode aligner suite v.1.5.2. (Codoncode Corp., Dedham, Ma, USA).

2.2.8 Microsatellite (MS) genotyping and eBURST analysis

Neospora caninum multilocus genotyping (MLG) based on nine MS markers (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21) was performed for the NC-SP1 isolate (DNA was derived from tachyzoites) under PCR conditions that had previously been described (Regidor-Cerrillo *et al.* 2013). These multiplex PCRs were performed on approximately 200 ng of DNA. *N. caninum* (Nc-Spain7) DNA was included in each batch of amplifications as a reference isolate. MS allele assignment was performed in accordance with the sizes determined by capillary electrophoresis and sequencing of the MS5, MS10 and MS7 markers was done as previously described (Regidor-Cerrillo *et al*. 2006; 2013). The size of the 6-FAM-labelled PCR products for all of the MSs was determined using a 48-capillary 3730 DNA analyser (Applied Biosystems) with Gene Scan-500 (LIZ) size standards (Applied Biosystems) and MS sequencing with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and a 3730 DNA analyser (Applied Biosystems) at the Genomic Unit of the Madrid Science Park, Spain. The sizes of the PCR products and sequences were analysed using the GeneMapper1 v3.5 software (Applied Biosystems) and BioEdit Sequence Alignment Editor v.7.0.1 (Copyright_ 1997–2004 Tom Hall, Ibis

Therapeutics, Carlsbad, CA, USA), respectively, as described previously (Regidor-Cerrillo *et al*. 2013).

The eBURST software was used to explore the closest genetically related genotypes of NC-SP1 in a dataset involving 81 *N. caninum* 9-MLGs from Argentine, Spanish, German and Scottish populations (Regidor-Cerrillo *et al.* 2013). The eBURST software generates networks composed of MLGs represented as dots, linked to their single-locus variants (SLV: 8 shared loci out of 9) and double-loci variants (DVL: 7 shared loci out of 9), line by line (Feil *et al*. 2004).

2.2.9 Animal ethics

All the animals were handled in accordance with protocols that had been approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil (CEUA no. 8634270114), following the National Research Council Guide for the Care and Use of Laboratory Animals.

2.3 RESULTS

2.3.1 Bioassay in a dog

DNA of *N. caninum* was detected in one out of four aliquots from the cattle brain.

Neospora-like oocysts were detected in dog feces on day 7 PI. The duration of oocyst shedding was 14 days and the number of oocysts produced during the patent period was 144500 oocysts. The average number of oocysts per gram of feces was 102 oocysts/g (a range of 2-946) (Supplementary Table S1). The unsporulated oocysts measured 9.85–12.20 (11.19 ± 0.45) µm x 10.28–12.42 (11.46 ± 0.47) µm.

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No clinical signs were observed and antibodies against *N. caninum* (IFAT < 50) were not detected in the dog serum during the period analysed (19 weeks).

2.3.2 Inoculation of the cattle brain in rodents and cell culture

The cell culture flask inoculated with the cattle brain tissue was examined for 2 months and no parasites were observed. Also, none of the gerbils seroconverted.

2.3.3 Infection of gerbils with oocysts and isolation in cell culture

All gerbils fed 200 oocysts seroconverted 14 days PI. The range of titres was from 100 to 25600 (Supplementary Fig. S1). The gerbils remained clinically healthy. The PCR analysis based on the Nc-5 gene showed that the parasite was distributed in the tissues of the 16 gerbils (Supplementary Table S2).

Successful isolation in cell culture occurred with the brain homogenate from a gerbil inoculated with 10^2 oocysts that was euthanized 78 days PI. The acid-pepsin digestion method had been used for this sample. Tachyzoites were observed 24 days PI in the cell culture, but the isolate had slow growth. Six tissue cysts from fresh brain homogenate of the same gerbil were viewed under a microscope and were found to measure 23.84 ± 4.14 µm x 25.69 ± 5.30 µm.

2.3.4 Identity of *Neospora*-like oocysts

DNA of *N. caninum* was detected on 6 of the 14 days of oocyst shedding (days 7, 8, 9, 10, 11 and 13 PI) using nested ITS-1 PCR. In the same fecal samples, using Np6 plus and Np21 plus primers, it was also possible to detect DNA of *N. caninum*.

The results based on ITS-1 nested PCR-RFLP indicated that only oocysts of *N. caninum* were present.

The final sequence deposited in GenBank®, under accession number KT581980, was 99% identical to homologous sequences of many *N. caninum* isolates available in the same database.

2.3.5 MS genotyping and genetic relationship

Alleles for each of the nine MS markers analysed were amplified and identified from a DNA sample obtained from NC-SP1 tachyzoites. No new alleles were detected, although comparison of the NC-SP1 MLG with the database of 81 MLGs showed that it has a unique and different genetic profile. In addition, the eBURST analysis detected absence of SLVs with NC-SP1. Only four DLVs (with variation in two MS markers) were found associated with the NC-SP1 MLG. These genetically closest MLGs involved three MLGs of Spanish and one MLG of Argentine origin (Supplementary Table S3, Figs. S2 and S3). In addition, comparison of NC-SP1 MLG with other MLGs of *N. caninum* isolates obtained in Brazil, the NC-Bahia (Gondim *et al.* 2001) and NC-Goiás (García-Melo *et al.* 2009) isolates, showed variation in three and four out of nine markers, respectively (Table 1).

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Table 1. Microsatellite genotyping of the *Neospora caninum* isolate NC-SP1 and comparison with the genetically closest isolates analysed and with other Brazilian isolates

			Microsatellite markers								
Isolates	**Host**	**Location***	**MS4**	**MS5**	**MS6A**	**MS6B**	**MS7**	**MS8**	**MS10**	**MS12**	**MS21**
NC-SP1 (BRA-15)	Cattle	BRA	13	19	15	12	9.1	13	6.14.10	16	6
NC-Bahia	Dog	BRA	13	13	15	12	9.1	14	5.14.9	16	6
NC-Goiás	Cattle	BRA	13	18	14	12	9.1	12	6.16.9	16	6
SP02-GAL-S-26	Cattle	SPA	13	19	15	12	14	13	6.14.9	16	6
SP05-GAL-W-40	Cattle	SPA	13	15	15	12	9.1	13	6.14.8	16	6
SP06-GAL-W-48	Cattle	SPA	13	14	15	12	9.1	13	6.14.8	16	6
ARG-04-E-1	Cattle	ARG	13	17	15	12	9.1	13	6.15.8	16	6

*BRA: Brazil; SPA: Spain; ARG: Argentina.

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2.4 DISCUSSION

Successful isolation of the NC-SP1 isolate of *N. caninum* from a naturally infected cattle was possible due to the bioassay in a dog. Attempts to isolate the parasite from the primary sample (cattle brain) in cell cultures and rodents were not successful in this study. Dogs are efficient experimental hosts for isolation of *N. caninum*, because they can ingest large amounts of animal tissues (Gondim *et al*. 2002). In our study, the dog ate approximately 390 g of brain, compared with 10 g used for cell cultures and rodents. However, not all laboratories have the proper facilities to house and use dogs as experimental models, and therefore standardized protocols to isolate *N. caninum* need to be improved. Dubey and Schares (2011) emphasized in their review that isolation of viable parasites is difficult and that finding DNA is not as valuable as isolating viable parasites.

In this report, the results related to the *N. caninum* bioassay in a dog corroborate previous findings. The number of oocysts excreted by the dog can be considered to be a medium quantity of oocysts for *N. caninum*, in comparison with other reports (Lindsay *et al*. 1999; Rodrigues *et al*. 2004). Gondim *et al*. (2002) reported variance in the quantity of oocysts shed by the dogs and they pointed out that the dogs shed more oocysts when they were fed with infected cattle tissues than when fed with infected mouse tissues. Furthermore, the dog remained clinically healthy and did not develop antibodies against *N. caninum*. These facts were also consistent with the findings from other studies (Basso *et al*. 2001; Gondim *et al*. 2005; Pena *et al*. 2007).

Gerbils are considered to be susceptible hosts for clinical neosporosis, but results are inconsistent (see Dubey *et al.* 2007). However, the stage of the parasite needs to be taken into consideration. In our study, none of the gerbils orally fed 1000 oocysts developed clinical signs, thus suggesting that the new isolate has low pathogenicity in gerbils. The capacity of isolates of *N. caninum* to induce clinical disease or death in the experimental models and the efficiency of *in vitro* proliferation are important features to be evaluated in virulence studies (Regidor-Cerrillo *et al*. 2011; Dellarupe *et al*. 2014). Nevertheless, the results from virulence studies should be evaluated with caution because the interpretation can change according to the hosts,

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laboratory conditions and biological characteristics. The NC-SP1 isolate grew slowly in the cell culture and did not have the capacity to destroy the monolayer of VERO cells. More efforts will be made to adapt the new isolate to cell culture.

The sequencing of the *N. caninum* ITS-1 region confirmed that the new isolate is a member of the species *N. caninum*. MS genotyping has the power to distinguish between individual organisms and this genetic analysis showed that NC-SP1 has a unique genetic profile, in comparison with the database of 81 *N. caninum* MLGs. In addition, the four genetically closest MLGs were from isolates that originated from aborted bovine fetuses in Spain and Argentina (Regidor-Cerrillo *et al*. 2013). Unfortunately, there is only a limited number of Brazilian isolates, and therefore NC-SP1 MLG could only be compared with two other isolates: NC-Bahia (Gondim *et al*. 2001) and NC-Goiás (García-Melo *et al*. 2009), isolates from a dog and a bovine, respectively.

In summary, our study presented the biological and genetic characterization of a new isolate of *N. caninum*, named NC-SP1, from an adult naturally infected cattle. New attempts to isolate *N. caninum* from domestic and wild animals would be important for improving the understanding of the biological and genetic diversity among *N. caninum* isolates in Brazil.

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SUPPLEMENTARY MATERIAL

Table S1. Excretion of *Neospora caninum* oocysts by the dog bioassayed during the experimental period

Days post inoculation	Amount of feces/day (g)	N. of oocysts/g	Total of oocysts/ day
1	110	11	1210
2	102	15	1530
3	107	151	16157
4	122	34	4148
5	95	946	89870
6	99	25	2475
7	110	27	2970
8	156	53	8268
9	140	23	3220
10	100	122	12200
11	139	11	1529
12	90	2	180
13	139	5	695
14	138	2	276

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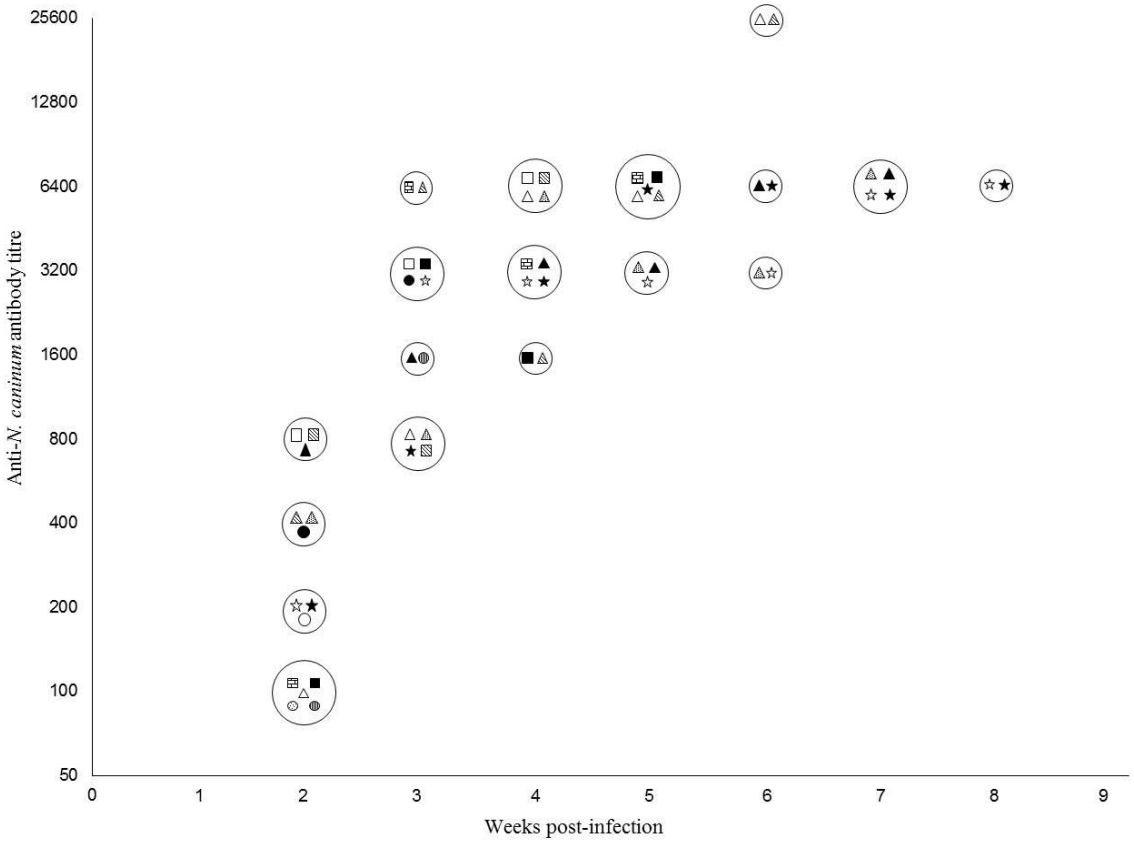

Fig. S1. Titres of anti-*N. caninum* IgG antibodies in fourteen gerbils that were orally challenged with 200 oocysts of the isolate NC-SP1. Two gerbils were euthanized seven days PI (IFAT < 50). Each gerbil is represented by a symbol inside the circles.

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Table S2. *Neospora caninum* tissue distribution in gerbils that were orally challenged with 200 oocysts of the isolate NC-SP1, based on DNA detection*

	Weeks post-inoculation							
Tissues collected	**1st**	**2nd**	**3rd**	**4th**	**5th**	**6th**	**7th**	**8th**
Blood								
Skeletal muscle								
Tongue								
Liver								
Spleen								
Diaphragm muscle	•							
Reproductive system	•							
Lung	•	•						
Intestine		•						
Heart		•						
Kidney			•					
Mesenteric lymph node	•	•	•	•				
Eye		•				•		
Spinal cord		•	•	•		•	•	•
Brain	•	•	•	•	•	•	•	•

* PCR assays using the primers Np6 plus and Np21 plus.

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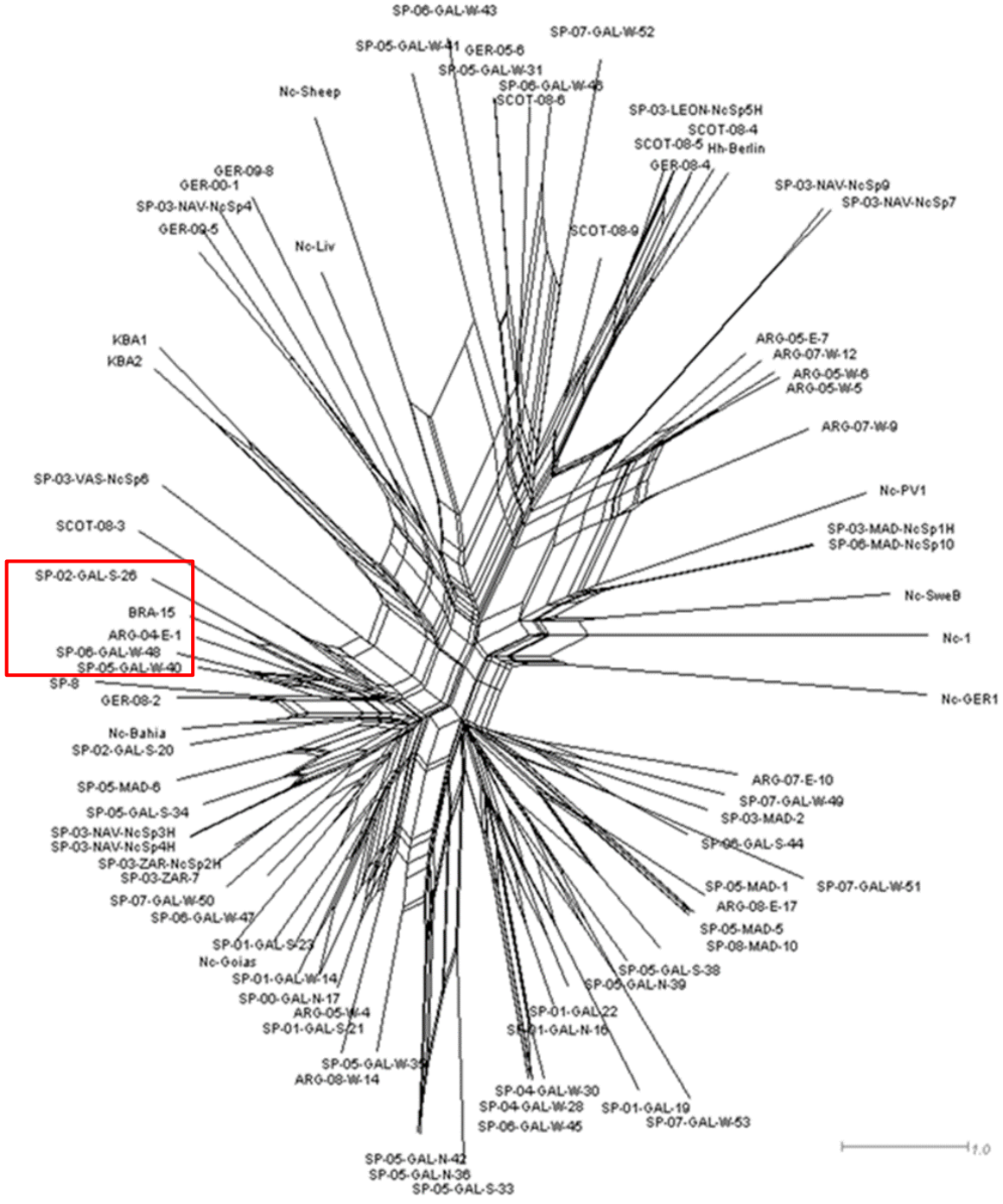

Fig. S2. Phylogenetic analysis showing genetic relationships of the *Neospora caninum* isolates and NC-SP1 isolate (BRA-15).

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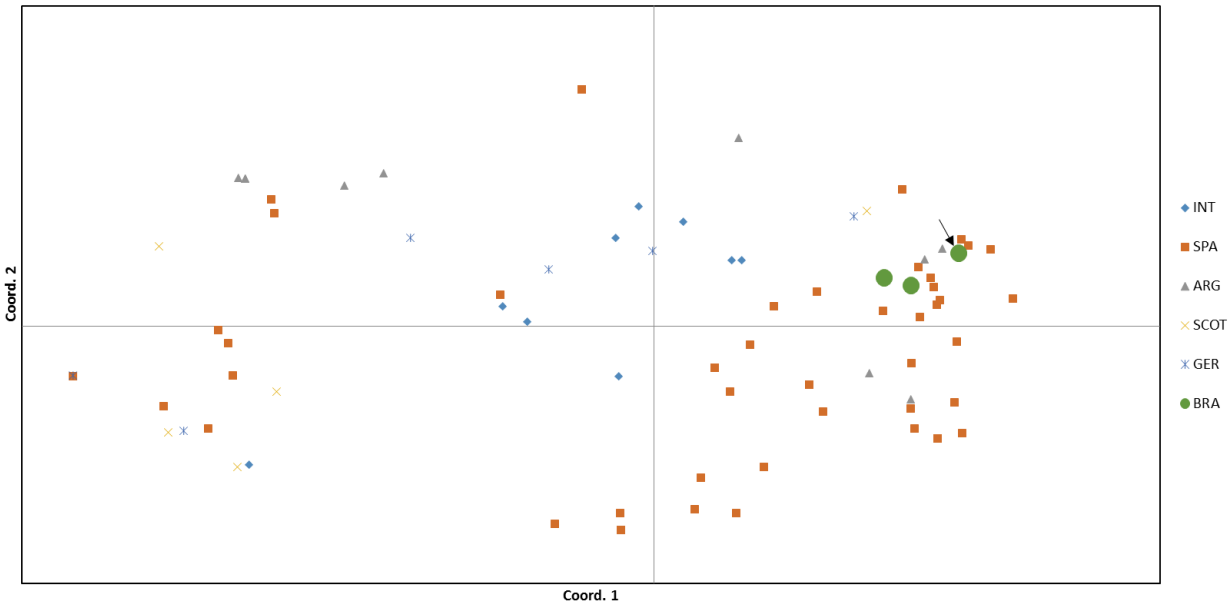
Table S3. Principal Coordinates Analysis (PCoA)

PCoA via Covariance matrix with data standardization				**No. of samples by population**					
				INT	**SPA**	**ARG**	**SCOT**	**GER**	**BRA**
Data Sheet	GD (2)								
Data Title	ALLELE FREQ								
No. Samples	82	**No. Pops.**	6	10	49	10	5	6	2

Percentage of variation explained by the first 3 axes			
Axis	**1**	**2**	**3**
%	15,28	8,38	7,01
Cum %	15,28	23,66	30,68



INT: Reference strains; SPA: Spain; ARG: Argentina; SCOT: Scotland; GER: Germany; BRA: Brazil.

Fig. S3. Population structure based on principal coordinate analysis (PCA).

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3. EXPERIMENTAL *Neospora caninum* INFECTION IN CHICKENS (*Gallus gallus domesticus*) WITH OOCYSTS AND TACHYZOITES OF TWO RECENT ISOLATES REVEALS RESISTANCE TO INFECTION

ABSTRACT

The importance of birds in the biological cycle of *Neospora caninum* is not clear. We report unsuccessful *Neospora* infection in chickens (*Gallus gallus domesticus*) using two isolates of *N. caninum*. In experiment #1, 30 White Leghorn chickens were orally inoculated with viable *N. caninum* oocysts (NC-SP1 isolate, 200 oocysts per bird) via the crop at 21 days of age. Groups of three birds were euthanized at intervals of 7 days (a total of 9 weeks) and one group was challenged with the same oocyst dose at 37 days p.i. and observed for 11 weeks. Blood samples were collected weekly, and sera were tested using IFAT. Chicken tissues were collected for PCR, quantitative PCR and immunohistochemistry. Two dogs approximately 45 days of age were fed with tissues from chickens euthanised at 138 and 159 days p.i. The results indicated that the chickens were resistant to neosporosis as revealed by failure to seroconvert, to detect parasite DNA or *N. caninum* antigen by IHC in inoculated bird tissues, and by no oocyst excretion by the dogs fed avian tissues. Similar results were obtained in experiment #2, in which 34 1-week old chickens were each s.c. inoculated with 100,000 tachyzoites of the NcWTDMn1 isolate of *N. caninum.* The chickens were euthanised on days 7, 15, 22, 28, 36 and 60 p.i. At necropsy, all tissues and serum from each bird were collected. All chickens remained asymptomatic, and *N. caninum* antigen was not detected by immunohistochemistry. Seven chickens euthanised at day 60 p.i. demonstrated low (1:25 dilution) levels of antibodies by using the *Neospora* agglutination test. Two 12-week-old dogs fed tissues pooled from 10 inoculated chickens euthanised day 60 p.i. did not excrete *N. caninum* oocysts. This investigation indicates that chickens are resistant to experimental infection by *N. caninum.*

Keywords: Neosporosis, Bioassay in dogs, Chickens, Intermediate hosts, Faeces, PCR

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3.1 INTRODUCTION

Neospora caninum is distributed worldwide and causes severe economic losses in cattle farming due to abortions; it is also known to cause clinical disease in several other hosts, especially dogs (Dubey et al., 2017). In the biological cycle of this protozoan, some canids (dogs, dingoes, coyotes and grey wolves) act as definitive hosts, excreting environmentally-resistant oocysts (McAllister et al., 1998; Gondim et al., 2004; King et al., 2010; Dubey et al., 2011). Viable parasites have been previously isolated from a variety of mammals (Dubey et al., 2017), which allows these species to be classified as intermediate hosts.

Since the description of *N. caninum* as a species in 1988 (Dubey et al., 1988), many studies have been conducted to clarify its biology and epidemiology, but the importance of birds in its biological cycle is uncertain (Dubey et al., 2017). Seroprevalence studies in free-range chickens (*Gallus gallus domesticus*) indicate that these animals are exposed to this parasite (Martins et al., 2011; Dubey et al., 2017). Experimental studies using tachyzoites have been conducted in domestic and wild birds but the results are not definitive (McGuire et al., 1999; Furuta et al., 2007; Mineo et al., 2009). The NC1 strain was the first isolate of this parasite and was used in most of these investigations; however, it has been passaged in animal models and cell culture several times since 1988, which draws attention to biological changes; for instance, Bartley et al. (2006) showed that tachyzoites of *N. caninum* (NC1) maintained for different lengths of time in cell culture can be more (low-passage parasites) or less (high-passage parasites) pathogenic to mice. Also, the variability in outcomes of experiments with NC1 isolate in cattle can be related to this (Dubey et al., 2017). The initial report that chickens are intermediate hosts of *N. caninum* was based on molecular findings (Costa et al., 2008), but attempts to isolate the parasite from seropositive chickens were unsuccessful (Gonçalves et al., 2012).

The objective of this study was to experimentally infect chickens with two *N. caninum* isolates that have gone through minimal passage, and by using two stages of the parasite and two routes of inoculation (orally with oocysts using NC-SP1 and

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tachyzoites, s.c., with NcWTDMn1) to study the kinetics of infection by *N. caninum* in these birds.

3.2 MATERIALS AND METHODS

Two experiments were performed. Experiment #1 was conducted in Brazil in 2014 and 2015. Experiment #2 was conducted in USA in 2009.

3.2.1 Experiment #1

3.2.1.1 Inoculum

Oocysts of *N. caninum* NC-SP1 used to infect chickens were collected and stored in 2% H_2SO_4 at 4°C as described previously (Oliveira et al., 2017). To remove H_2SO_4 from the inoculum, oocysts were washed twice with Hank's balanced salt solution (HBSS) buffer, (GIBCO®, USA) by centrifugation at 1,500 *g* for 10 min, and the pellet was resuspended in aqueous 0.85% NaCl solution (saline). The estimated number of oocysts in the inoculum was determined according to the protocol described by Pena et al. (2007). Oocysts were 4 months old when they were inoculated into chickens and gerbils.

Two gerbils (*Meriones unguiculatus*) were orally inoculated with 200 oocysts/gerbil on the same day as chicken inoculation to confirm the viability of the oocysts used in the experimental infection of the chickens. For 11 weeks p.i., blood samples were collected weekly, and sera were tested using IFAT (Camargo, 1974). At 119 days p.i., the two gerbils were euthanised, and samples of tissues, including brain, heart, lung, spleen, liver, kidney, mesenteric lymph node, tongue, eye, spinal cord, thigh muscle, reproductive tract and blood, were collected for molecular analysis. The

brain of each gerbil was removed, homogenised and digested in a pepsin-acid solution (1.3 g pepsin, 2.5 g NaCl, 3.5 mL HCl and 250 mL distilled water) for 1 h at 37°C (Dubey, 1998). After digestion, the homogenate was washed three times with HBSS buffer by centrifugation at 1,500 *g* for 10 min, and the pellet was resuspended in RPMI 1640 medium. For each brain homogenate, one gerbil was inoculated i.p. (500 µL/animal); in addition, a flask containing a monolayer of Vero cells was inoculated (500 µL/flask).

3.2.1.2 Chickens

White Leghorn lineage chickens were acquired at 1 day of age (Biovet® Laboratório, São Paulo, Brazil) and kept in circular pens under controlled temperature and ventilation until 21 days of age. Water and food were provided ad libitum throughout the experimental period. The feed was formulated according to the age group of the birds and did not include coccidiostats. Throughout the experimental phase, the birds were housed in groups of three and were kept in cages suitable for the species.

Thirty 21-day-old birds were inoculated via the crop with 200 oocysts per bird and randomly distributed into 10 groups with three birds each (G1 to G10). G10 was challenged at 37 days p.i. with the same oocyst dose. An uninoculated control group containing two birds was maintained during the experiment. Blood was collected weekly from all birds via the brachial vein, and the sera were tested for *N. caninum* antibodies (see section 3.2.1.3). Groups of birds were euthanised at intervals of 7 days; therefore, for groups G1 to G9, euthanasia occurred at the following times: 7, 14, 21, 28, 35, 42, 49, 56 and 63 days p.i., respectively. Group G10 was euthanised 45 days after the second inoculation. The control group was euthanised at 103 days p.i. During necropsy, fragments of organs including the brain, heart, lung, liver, spleen, kidney, eye, tongue, thigh muscle, pectoral muscle, ventricle, reproductive tract, intestine, thymus, spinal cord and whole blood were collected separately from each bird for both

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molecular analysis and immunohistochemistry (IHC) except for spinal cord and whole blood for IHC.

For each group of birds, brain fragments were pooled and digested in 0.05% trypsin at 37°C for 1 h (Dubey et al., 2013a). After digestion, the homogenate was washed three times with HBSS buffer by centrifugation at 1,500 *g* for 10 min, and the pellet was resuspended in an antibiotic-antimycotic solution (Vitrocell Embriolife®, Brazil), and two gerbils were inoculated i.p. (500 µL/animal). Two months p.i., the gerbils were euthanised, blood was collected by cardiac venipuncture followed by harvest of the brain for molecular analysis, and serum samples were tested by IFAT.

3.2.1.3 Serology

Serum samples from birds, gerbils, and dogs were tested by IFAT for anti-*N. caninum* antibodies, and the established cut-off points used were dilutions of 1:5 (birds) and 1:50 (gerbils and dogs). *Neospora caninum* tachyzoites derived from cell culture of the NC1 isolate (Dubey et al., 1988) were used as the antigen. Anti-chicken IgY (Sigma-Aldrich®, USA), anti-gerbil IgG (Immunology Consultants Laboratory® Inc., USA) and anti-dog IgG (KPL® Inc., USA) conjugates labelled with FITC were used. Negative and positive controls were added to all slides.

Serum samples from IFAT-positive birds were also tested using immunoblots (IB) according to the protocol described by Schares et al. (2010). The serum samples were diluted at 1:10, and purified p38 antigen of *N. caninum* (Schares et al., 2000) and peroxidase-labelled anti-chicken (IgY) conjugate (affinity purified goat [H+L], Jackson ImmunoResearch Laboratories, USA) at 1:1000 dilutions were used. The relative molecular mass was determined by comparison with the standard LMW-SDS Marker. As positive and negative controls, bovine sera were added and peroxidase-labelled anti-bovine conjugate was used (IgG [H+L], Jackson ImmunoResearch Laboratories, USA).

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3.2.1.4. Immunohistochemistry

Tissue samples collected from chickens were fixed in 10% buffered formalin for 24-48 h and stored in 70% ethanol. Five µm histological sections were deparaffinised and rehydrated and then subjected to antigenic recovery using citrate buffer (pH 6.0) for 3 min in a pressure cooker. Endogenous peroxidase blocking was performed by immersing the slides in 4% hydrogen peroxide for 20 min. An anti-*N. caninum* polyclonal antiserum produced in mice was used (1:200), and a commercial detection kit (NovoLink Polymer Detection System, Leica Biosystems®, Germany), and the chromogen 3,3′-diaminobenzidine (DAB, Dako®, Denmark) was used to reveal the reaction. Counter-staining was performed with Harris haematoxylin. Slides containing positive (brain of BALB-c mouse) and negative (tissues of non-infected BALB-c mouse) controls were processed simultaneously with the tissue samples. Presence or absence of *N. caninum* in the bird tissues was determined by a qualitative evaluation.

3.2.1.5 DNA extraction and PCR

DNA from the tissues of birds and gerbils was extracted using the commercial DNeasy Blood and Tissue kit (Qiagen® Inc., USA) according to the manufacturer's recommendations. Faecal samples from dogs were first subjected to six cycles of freezing in liquid nitrogen for 2 min and thawing at 37°C, and DNA was extracted with the commercial QIAamp DNA Stool Mini Kit (Qiagen® Inc.) according to the manufacturer's recommendations. Negative DNA extraction controls (ultrapure water) were included after every 12 tissue samples processed for DNA extraction.

The Np21 plus and Np6 plus primer pair (gene pNc5 of *N. caninum*) was used for conventional PCR according to the protocol of Müller et al. (1996); all reactions were done with final volume of 25 µL, and 2 µL of DNA were used. The amplified products were visualized by electrophoresis in 2% agarose gel and stained with SYBR® Safe DNA Gel Stain (Invitrogen™, USA).

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For real-time quantitative PCR (qPCR), the target was also the pNc5 gene of *N. caninum,* and the protocol used was that described by Neto et al. (2011), with a change of the fluorophores in the probe [6-carboxyfluorescein (6-FAM™) at the 5′ end and auxiliary internal Zen™ quencher and Iowa Black dark quencher® (IBFQ) at the 3′ end] (Integrated DNA Technologies, IDT®, USA). The assays were conducted with a final volume of 20 µL, and 2 µL of DNA were used; the DNA samples were assayed in duplicate, and the reaction mixture for qPCR used was 5x Hot FIREPol® Probe Master Mix (Solis BioDyne, Estonia).

Additionally, the qPCR performance for the pNc5 gene was verified using DNA from tachyzoites and oocysts. For that, DNA from $1x10^6$ tachyzoites (NC1 isolate) was extracted as described above and resuspended in 100 µL of Tris-EDTA (TE) buffer (concentration of $1x10^5$ equivalent genome per µL). This suspension was diluted to $1x10^{-1}$ using 10-fold serial dilution. DNA from oocysts (NC-SP1 isolate) was obtained from 500, 250, 100, 10, 5 and 0 oocysts and resuspended in 50 µL of TE buffer. The qPCR assays were performed using 2 µL of DNA.

For the endogenous control, primers and a probe targeting the cytochrome *b* (cytb) gene of the chickens were used as described by Dooley et al. (2004); the probe used was labelled with hexachlorofluorescein (HEX™) at the 5′ end and the auxiliary internal Zen™ quencher and IBFQ® at the 3′ end (IDT®). For the endogenous control used for the dog faeces samples, the SYBR® Green system was used to target the GAPDH gene, and the primers used were described by Leutenegger et al. (1999). The assays were conducted with a final volume of 20 µL, containing Power SYBR® Green PCR Master Mix (1X) (Applied Biosystems, Life Technologies, USA), 600 nM of each primer and 2 µL of DNA. The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min; subsequently, a melting curve was performed (95°C for 10 s, 60°C for 10 s and 95°C for 10 s). The endogenous controls and the target gene, *N. caninum* pNc5, were tested separately in the qPCR assays on a 7500 Real-Time PCR System (Applied Biosystems).

The DNA samples from the dog faeces and bird tissues were tested by both PCR and qPCR. The DNA obtained from the gerbil tissues was tested using PCR only.

3.2.1.6 Bioassay in dogs

Tissues of two groups of three inoculated birds each (G11 and G12) were used for the bioassay in dogs. The birds from G11 and G12 were inoculated at 7 and 21 days-of-age, respectively. Blood was collected weekly, and the serum samples were tested by IFAT as previously described in section 3.2.1.3. Two dogs of unspecified, mixed breed (dog 1, a male, and dog 2, a female), approximately 45 days-of-age and seronegative for *N. caninum* (IFAT <50), were used. The dogs were donated by their owner to be used in the experiment. Prior to the bioassay, the dogs were treated with fenbendazole (50 mg/kg/day) for 5 days. The animals were administered methylprednisolone acetate (Depo-Medrol®, Pfizer, Belgium) i.m. using the following protocol: 50 mg on days -7, -6 and 0; 25 mg on days 1 and 2; and 12.5 mg on days 3 and 4 of the bioassay. The immunosuppression protocol was used to increase the chance of oocyst shedding by the dogs (Lindsay et al., 1999). The dogs had never before ingested tissue from other animals and were fed a commercial dry dog food throughout the experiment. Both dogs were healthy at the end of the experiment and they were adopted.

Chickens from G11 were euthanised at 138 days p.i., and those from G12 at 159 days p.i. Samples of brain, heart, lung, liver, spleen, kidney, thigh and pectoral muscles, reproductive tract and spinal cord were collected separately from each bird for molecular analysis. Tissues used in the bioassay were skeletal musculature, brain, heart and spinal cord. Virtually all of the skeletal muscles were removed from the chicken carcasses and cut into small pieces; brain, heart and spinal cord were offered to the dogs on the first day of the bioassay, and the skeletal musculature was divided over 3 days. The tissues were stored at 4°C during the bioassay. Dog 1 ingested the tissues corresponding to G11, while dog 2 ingested the tissues from G12.

Faecal samples from each dog, separately, were collected daily (0 to 30 days p.i.) and examined using a standard flotation in sucrose solution technique (Ogassawara et al., 1986) to detect *N. caninum* oocysts. First, 5 g of faeces were diluted in distilled water, filtered through gauze (Gondim et al., 2002), added to the

sucrose solution (1,203 g/cm^3), centrifuged at 1,500*g* for 10 min and examined under a compound microscope at 400x magnification.

Another two 5 g aliquots of faeces were processed as described previously, and after flotation of these samples in the sucrose solution, 1 mL of the supernatant was collected, washed three times with TE buffer and centrifuged at 3,000*g* for 10 min to remove the sucrose solution. The pellet was then resuspended in 250 µL of TE and subjected to DNA extraction.

The design for the experimental infection of chickens with *N. caninum* oocysts is illustrated in Fig.1 (Experiment #1).

3.2.2 Experiment #2

3.2.2.1 Inoculum

Tachyzoites of the sixth passage of NcWTDMn1 isolated in 2009 from the brain of an aborted white-tailed deer foetus (Dubey et al., 2013b) were used to infect chickens. For this, tachyzoites were grown in CV1 cells. Infected cell cultures were scraped, passaged through 27 gauge needles, counted, and suspended to 200,000 tachyzoites/mL of the tissue culture medium. The viability of the inoculum used to inoculate chickens was ascertained by bioassay in three interferon gene gamma knock out (KO) mice; the inoculated KO mice died of neosporosis within 2 weeks.

3.2.2.2 Chickens

One-week-old Sexsal egg-layer chickens (n = 44, obtained from Moyer's hatchery, Quakerstown, PA, USA) were used. Each of 34 chickens was inoculated s.c. with 100,000 (0.5 mL inoculum) tachyzoites and 10 chickens were kept as controls.

The chickens were euthanized on days 7 (three inoculated, three controls), 15 (six inoculated), 22 (six inoculated), 28 (six inoculated), 36 (three inoculated, three controls) and 60 (10 inoculated, four controls) of the experiment or p.i.

All chickens were euthanised by cervical dislocation and blood was collected by cutting the neck. Samples of all tissues including the brain, heart, lungs, liver, kidneys, small intestine, eyes, and bursa were fixed in 10% buffered formalin and processed for histology. Sections were examined microscopically after staining with H&E and IHC was performed as described (Dubey et al., 2017).

Tissues of 10 inoculated chickens euthanised day 60 p.i. were used for bioassay in two dogs and four KO mice. For this, portions of brain, heart, and muscles from legs and breast were pooled and approximately 500 g of the pooled tissues were fed to each of the two dogs over a period of 3 days. The dogs were 12 weeks old, laboratory raised, seronegative for *N. caninum*, and had not been previously fed meat. Faeces of dogs were examined microscopically for oocysts 3-30 days p.i., essentially as described in experiment #1. Additionally, portions of brains of dogs were homogenised in saline, centrifuged, and aliquots were inoculated subcutaneously into four KO mice. Tissues of KO mice were examined for neosporosis as described (Dubey et al., 2013b).

Sera of chickens, dogs, and mice in experiment #2 were tested for *N. caninum* antibodies using the mouse-derived whole *N. caninum* tachyzoites (*Neospora* agglutination test, NAT) as described by Romand et al. (1998). Sera were tested at 1:25 and 1:100 dilutions.

The design for the experimental infection of chickens with *N. caninum* tachyzoites is illustrated in Fig. 1 (Experiment #2).

3.2.3 Statistical analysis

The 95% confidence interval (95% CI) for each experiment was calculated using an exact Binomial approach. Calculations were made in R version 3.4.1 (R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for

Statistical Computing, Vienna, Austria. URL http://www.r-project.org/), using the "stats" package.

3.2.4 Animal ethics

All animals were handled according to the protocols approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil, and the Beltsville Agricultural Research Center Animal Care Committee, United States Department of Agriculture, Beltsville, Maryland, USA.

Oliveira, S., Aizawa, J., Soares, H.S., Chiebao, D.P., Castro, M.B., Hora, A.S., Lopes, M.G., Schares, G., Jenkins, M.C., Kwok, O.C.H., Dias, R.A., Gennari, S.M., Dubey, J.P., Pena, H.F.J., 2017. Experimental *Neospora caninum* infection in chickens (*Gallus gallus domesticus*) with oocysts and tachyzoites of two recent isolates reveals resistance to infection. Int. J. Parasitology in press.

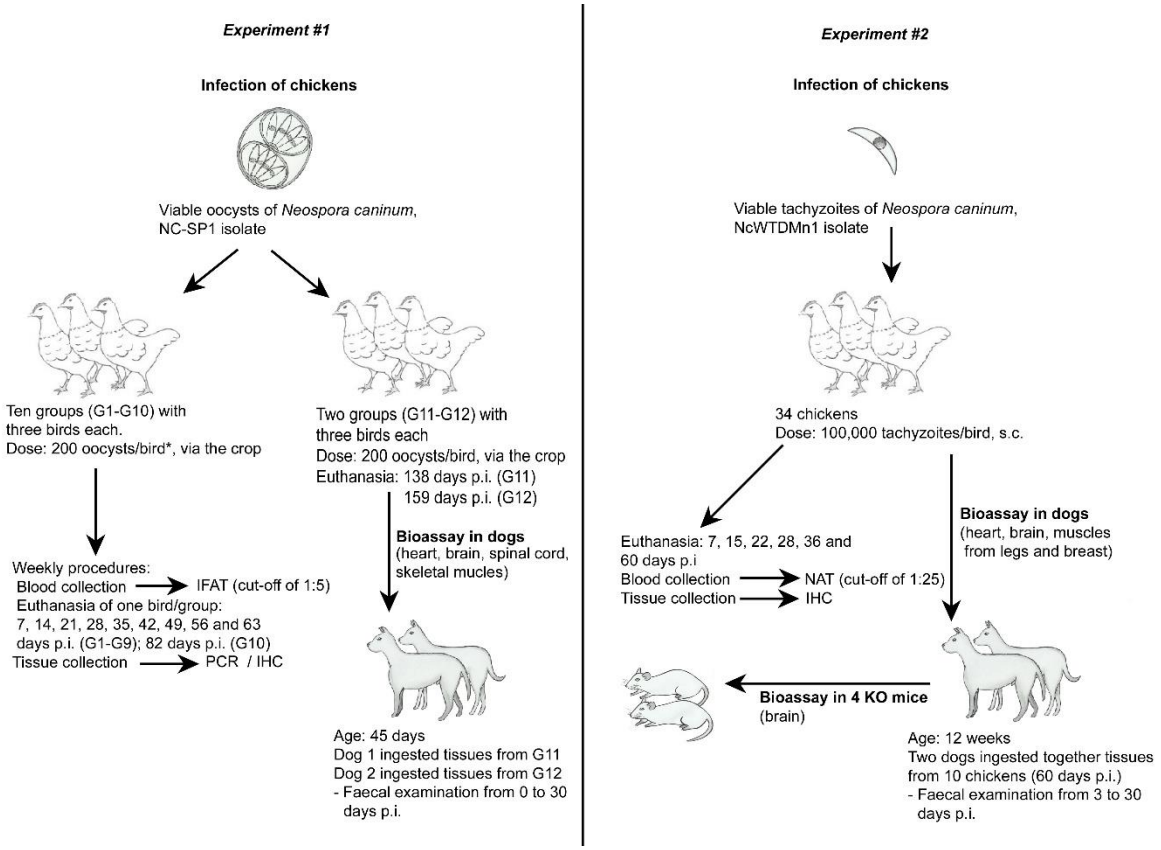

Fig. 1. Experimental infection of chickens with *Neospora caninum* isolates. Experiment #1: oral inoculation of chickens with viable NC-SP1 oocysts; tissues collected for immunohistochemistry and/or PCR: brain, heart, lung, spleen, liver, kidney, eye, tongue, thigh and pectoral muscles, ventricule, reproductive tract, intestine, thymus, spinal cord and whole blood; *G10 was challenged with 200 oocysts/bird at 37 days p.i. Experiment #2: s.c. inoculation of chickens with viable NcWTDMn1 tachyzoites; tissues collected for IHC: brain, heart, lungs, liver, kidneys, small intestine, eyes and bursa. G, group; KO, knockout; IHC, immunohistochemistry; NAT, *Neospora* agglutination test.

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3.3 RESULTS

3.3.1 Experiment #1

3.3.1.1 Viability test for *N. caninum* oocyst

The two gerbils inoculated with 200 oocysts/gerbil seroconverted at 14 days p.i. and had high anti-*N. caninum* antibody titres (ranging from 400 to 12,800) during the observation period (11 weeks). At the time of euthanasia (119 days p.i.), sera of these gerbils had IFAT titres of 1600 to 6400 (Supplementary Fig. S1). Molecular tissue analysis by PCR detected *N. caninum* DNA only in the brains of these gerbils. These results confirmed the viability of the *N. caninum* oocysts on the day that the chickens were infected.

The gerbils inoculated with the brain homogenate of the first two gerbils seroconverted within 14 days p.i.; however, in the cell culture the presence of *N. caninum* tachyzoites was not observed although maintained for 2 months, probably because NC-SP1 isolate has the feature of growing slowly in cell culture and of producing few tissue cysts in gerbils as observed by Oliveira et al. (2017).

3.3.1.2 Experimental infection of chickens

All chickens were clinically healthy and remained seronegative for *N. caninum* (titres <5) over the course of the experiment. *Neospora caninum* was not detected by IHC in any chicken tissues. Similarly, *N. caninum* DNA was not observed in bird tissues tested by PCR and qPCR; all DNA samples from the bird tissues amplified for the cytb gene (endogenous control; qPCR), indicating the absence of PCR inhibitors and the

successful extraction of the genetic material from the sample. The qPCR was able to detect equivalent genome of two tachyzoites and DNA equivalent to five oocysts.

The gerbils inoculated with the brain homogenate from each bird group did not seroconvert for up to 2 months p.i., and *N. caninum* DNA was not detected in the brain of the gerbils. The two birds of the control group were negative in all analyses.

3.3.1.3 Bioassay in dogs

One bird from each group died during the experiment of unknown causes (a G11 bird at 133 days p.i. and a G12 bird at 118 day p.i.), and *N. caninum* was not detected in the tissues of these birds using PCR, qPCR or IHC.

Of the six birds used for bioassay in dogs, five were seronegative for *N. caninum* (titre <5). Only one bird from G12 (bioassay of dog 2) had anti-*N. caninum* antibodies on days 21, 28, 34 and 41 p.i., with titres of 20, 20, 10 and 10, respectively, by IFAT; the same samples were positive in an IB at a 1:10 dilution.

Each dog ingested tissues corresponding to two birds; dog 1 ingested approximately 350 g of tissue (G11), and dog 2 ingested approximately 430 g of tissue (G12). In the coproparasitological examinations of the dogs, *N. caninum* oocysts were not observed. At the end of the experiment, the dogs remained seronegative for *N. caninum* (titres < 50).

Neospora caninum DNA was not found in the bird tissues nor in the dog faecal samples (PCR and qPCR), but all DNA samples amplified to the respective endogenous controls (genes cytb and GAPDH; qPCR).

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3.3.2 Experiment #2

All chickens remained clinically normal. Antibodies to *N. caninum* were not detected in a 1:25 dilution of serum of chickens 7-36 days p.i. nor in control chickens. Seven of 10 chickens euthanised at day 60 p.i. had low NAT titres at 1:25 dilution. Lesions and *N. caninum* were not detected in histological sections of tissues of any chickens. The two dogs fed tissues from inoculated chickens did not excrete oocysts; both dogs did not develop *N. caninum* antibodies (NAT, 1:25) when the experiment was terminated 30 days after dogs were fed chicken tissues. The KO mice inoculated with chicken brain homogenate remained negative for *N. caninum*.

3.3.3 Statistical analysis

Even though no *N. caninum*-positive chicken was found, the 95% CI ranged from 0 to 11.6% for experiment #1 and from 0 to 10.3% for experiment #2.

3.4 DISCUSSION

We believe this is the first study of experimental oral infection of chickens using the natural route of infection with *N. caninum* oocysts (natural stage of ingestion) as the inoculum. Chickens in their natural environment seek food directly from the soil; therefore, the most important route of infection is believed to be the ingestion of sporulated oocysts found in the environment. For *Toxoplasma gondii*, a protozoan which is ancestrally and morphologically close to *N. caninum*, domestic chickens were shown to be excellent indicators of environmental contamination by oocysts and are widely used for isolation of the agent (Dubey et al., 2008; Feitosa et al., 2016). In experiment #1, the oocyst dose used as the inoculum was chosen based on the

knowledge that the definitive hosts of *N. caninum* do not excrete large numbers of oocysts (Gondim et al., 2002; Rodrigues et al., 2004).

In seroprevalence studies, anti-*N. caninum* antibodies are frequently found in free-range chickens from different parts of the world, with varying results. Martins et al. (2011) reported a seroprevalence of 39.5% (524/1324, IFAT) in chickens from different countries of the Americas, whereas Costa et al. (2008) reported 23.5% (47/200, IFAT) seropositive chickens in the state of Bahia, Brazil. For wild birds, Mineo et al. (2011) tested sera from 294 captive and free-range birds from 17 species and nine orders from Brazil, and anti-*N. caninum* antibodies were not detected (IFAT). Experimental studies with birds using tachyzoites administered systemically demonstrated seroconversion of the birds, followed by a marked decline in antibody titres until completely undetectable (Furuta et al., 2007; Mineo et al., 2009; Oliveira et al., 2013). In our study, of the 36 chickens orally inoculated with viable oocysts, only one bird had detectable anti-*N. caninum* antibodies, and the presence of these antibodies were observed for 4 weeks. Additionally, chickens inoculated with tachyzoites in experiment #2 remained seronegative or had very low titres.

The presence of *N. caninum* DNA has been reported in tissues of different species of wild birds (Gondim et al., 2010; Darwich et al., 2012; Salant et al., 2015; Rocchigiani et al., 2017) and free-range chickens (Costa et al., 2008; Gonçalves et al., 2012; Romero et al., 2016); in all studies, *N. caninum* DNA was found in the brain and/or heart of the birds (reviewed in Dubey et al., 2017). The presence of the parasite's DNA in the bird tissues does not exclude the necessity of isolating viable parasites from naturally infected birds (Donahoe et al., 2015; Dubey et al., 2017). For this reason, Gonçalves et al. (2012) performed a bioassay in KO mice for free-range chickens seropositive for *N. caninum* but they were not able to isolate the parasite.

In other studies, several experiments were conducted using different bird species in an attempt to understand the dynamics of *N. caninum* infection, and a variation in susceptibility to infection amongst species was observed. Pigeons (*Columba livia*) seem to be more susceptible to infection by *N. caninum* (McGuire et al., 1999; Mineo et al., 2009). By contrast, quail (*Coturnix japonica*) and zebra finches (*Poephila guttata*) are more resistant (McGuire et al., 1999; Oliveira et al., 2013). In

other experimental studies with chickens, only transient *N. caninum* infection was demonstrated (Furuta et al., 2007; Munhoz et al., 2014).

Differences in pathogenicity amongst *N. caninum* isolates were reported (Rojo-Montejo et al., 2009; Dellarupe et al., 2014). NC-SP1 isolate was obtained from the brain of an asymptomatic bovine and seemed to be non-pathogenic for gerbils; none of the gerbils orally inoculated with 1000 oocysts developed clinical signs and, in cell culture, the isolate grew very slowly and did not have the ability to destroy the monolayer of Vero cells (Oliveira et al., 2017). However, NcWTDMn1, isolated from the brain of an aborted white-tailed deer foetus, was able to kill the KO mice inoculated during the bioassays, and several tachyzoites were harvested from cell culture (Dubey et al., 2013b). It is clear that these isolates behaved differently in animal models and cell culture, however, any conclusion about pathogenicity should be made with caution.

In our experiment, *N. caninum* was not found in the avian tissue, either through IHC, PCR or qPCR. qPCR was used as an additional tool to confirm the presence or absence of *N. caninum* in bird tissue and dog faecal samples. Serologically (IFAT and immunoblot), only one bird had detectable anti-*N. caninum* antibodies. Seropositivity indicates contact with the agent but not necessarily the establishment of infection. Tissues from this seropositive chicken were bioassayed in dogs, which could increase the possibility of parasite recovery if the bird was actually infected since dogs are excellent experimental models for *N. caninum* isolation and are able to ingest large amounts of tissue during the bioassay (Gondim et al., 2002). The four dogs in experiments #1 and 2 ingested approximately 350 g of tissue each but did not excrete *N. caninum* oocysts. Throughout the experiment, the chickens were fed commercial feed formulated according to their age requirements and without the addition of coccidiostats. This precaution was taken because drugs possibly could affect the life stages of *N. caninum.*

Calculations of sample power and confidence intervals for zero inflated data are helpful in prevalence studies. Even with the use of small samples and finding no infection by *N. caninum*, the confidence intervals for both experiments were small, showing that if infection was found, it would happen in a small number of chickens.

Results of the present study affirm that chickens are resistant to experimental infection by *N. caninum* oocysts (NC-SP1) and tachyzoites (NcWTDMn1). NC-SP1 and

NcWTDMn1 are recently isolated strains, with confirmed different behaviour, but strain differences did not play a role in inducing infection of chickens with *N. caninum* in the present study. Reasons for this resistance are not known. It seems possible that the higher body temperature in birds prevents the establishment of a viable *N. caninum* infection and a recent report on in vitro experiments support this hypothesis (Rezende-Gondim et al., 2017). The characterisation of a species as an intermediate host in the *N. caninum* life cycle should, whenever possible, be based on the isolation of viable parasites.

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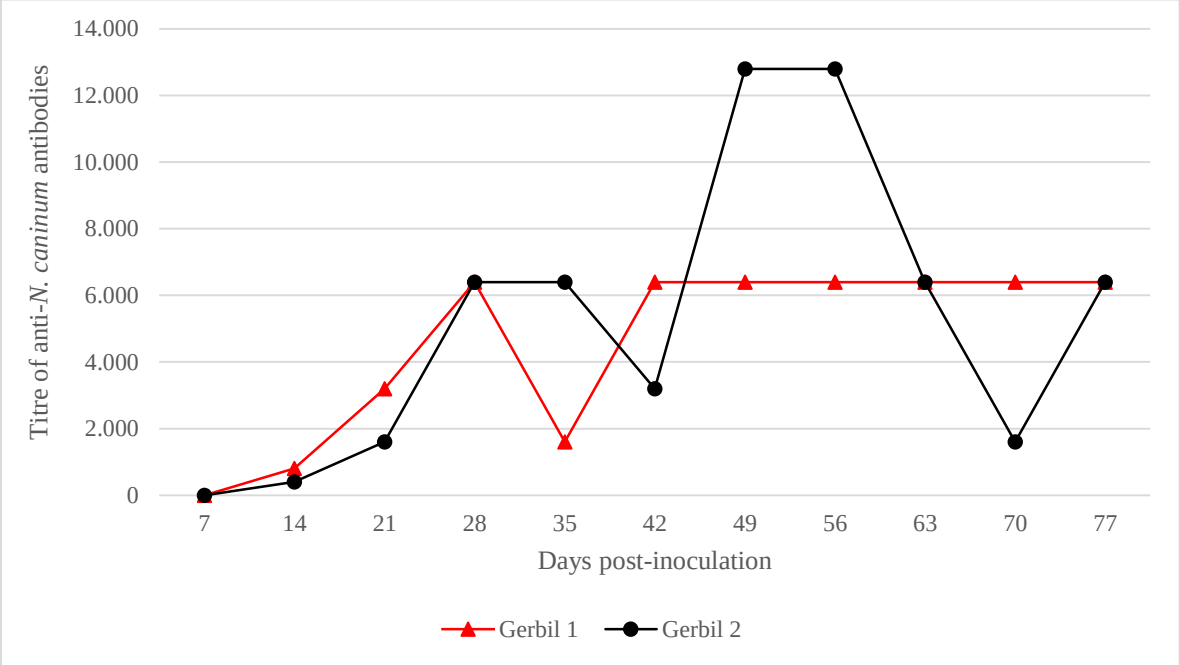
SUPPLEMENTARY MATERIAL



Supplementary Fig. S1. Titre of antibodies against *Neospora caninum* in gerbils orally inoculated with oocysts of the NC-SP1 isolate.

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4. CONCLUSIONS

- Nowadays, the use of animals in scientific and medical research, particularly with species affectively close to human beings such as dogs, has been a subject of debate in many levels of society. Nevertheless, in some experiments it is impossible to replace the animals, for example, in the two experiments carried out with *N. caninum* in which the purpose was to elucidate aspects of the biology of the parasite.
- The isolation of viable parasites is always the first option to define a species as an intermediate host of *N. caninum*, and the interpretation of finding DNA in tissues or antibodies in sera should be made carefully.
- The designed experimental infection pointed out that chickens are resistant to infection by *N. caninum* oocysts.
- We can assume that, in nature, if there is a possibility of infection by *N. caninum* in chickens, this occurrence should be very low and would not have a major impact on the biological cycle of *N. caninum*.

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