RAFAEL CARNEIRO COSTA

The Study of pathogenicity of two *Neospora caninum* strains isolated from goats and its genetic correlation with other strains by multilocus microsatellite genotyping

São Paulo
2020

Rafael Carneiro Costa

Estudo de patogenicidade de duas cepas de *Neospora caninum* isoladas de caprinos e sua correlação genética com outras cepas por genotipagem de microssatélites

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Departamento
Patologia

Área de concentração
Patologia Experimental e Comparada

Orientador
Dr. Paulo César Maiorka

São Paulo
2020

DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

T. 3905
FMVZ

Costa, Rafael Carneiro
The study of pathogenicity of two *Neospora caninum* strains isolated from goats and its genetic correlation with other strains by multiloccus microsatellite genotyping / Rafael Carneiro Costa. – 2020.
77 f. : il.

Título traduzido: Estudo de patogenicidade de duas cepas de *Neospora caninum* isoladas de caprinos e sua correlação genética com outras cepas por genotipagem de microssatélites.

Tese (Doutorado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Patologia, São Paulo, 2020.

Programa de Pós-Graduação: Patologia Experimental e Comparada.
Área de concentração: Patologia Experimental e Comparada.
Orientador: Prof. Dr. Paulo César Maiorka.
Coorientadora: Profa. Dra. Mary Suzan Varaschin.

1. Apicomplexa. 2. Abortos. 3. Bioensaio. 4. Microssatélites. I. Título.

Ficha catalográfica elaborada pela bibliotecária Maria Aparecida Laet, CRB 5673-8, da FMVZ/USP.

ERRATA

Costa, R. C. **The study of pathogenicity of two *Neospora caninum* strains isolated from goats and its genetic correlation with other strains by multiloccus microsatellite genotyping**. 2020. 74 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

Página	Onde se lê	Leia-se
FICHA CATALOGRÁFICA	77 f.	74 f.

CERTIFICADO

Certificamos que a proposta intitulada "Isolamento e estudo de patogenicidade de uma nova cepa de Neospora caninum isolada de caprinos", protocolada sob o CEUA nº 7161170516 (ID 002643), sob a responsabilidade de **Paulo César Maiorka** *e equipe; Rafael Carneiro Costa; Mary Suzan Varaschin* - 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 15/08/2016.

We certify that the proposal "Isolation and pathogenicity evaluation of a new Neospora caninum strain from naturally infected goats ", utilizing 48 Gerbils (48 males), 96 Isogenics mice (96 males), protocol number CEUA 7161170516 (ID 002643), under the responsibility of **Paulo César Maiorka** *and team; Rafael Carneiro Costa; Mary Suzan Varaschin* - 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 08/15/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 07/2016 a 02/2020 Área: Patologia Experimental E Comparada

Origem:	Não aplicável biotério						
Espécie:	Gerbil	sexo:	Machos	idade:	6 a 8 semanas	N:	48
Linhagem:	heterogênicos			Peso:	25 a 45 g		
Origem:	Biotério do Departamento de Patologia da FMVZ USP						
Espécie:	Camundongos isogênicos	sexo:	Machos	idade:	6 a 8 semanas	N:	96
Linhagem:	Balb c			Peso:	15 a 30 g		

Local do experimento: laboratório de patologia animal FMVZ-USP

São Paulo, 29 de novembro de 2019

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

Roseli da Costa Gomes
Secretária
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

FOLHA DE AVALIAÇÃO

Autor: Costa, Rafael Carneiro
Título: Estudo de patogenicidade de duas cepas de Neospora caninum isoladas de caprinos

Tese apresentada ao Programa de Pós-Graduação em Patologia Experimental e Comparada da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

Data: _____/_____/_____

Banca Examinadora

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

Instituição:_____ Julgamento:_____

Agradecimentos

À FAPESP, pela concessão da bolsa de estudos de doutorado (processo 2016/05010-2) e a bolsa de estágio em pesquisa no exterior (processo 2018/16546-6).
Ao Professor Paulo C. Maiorka, pela oportunidade e orientação durante todo o processo.
À Professora Mary S. Varaschin, pela contribuição na execução do projeto.
Aos pesquisadores Frank Katzer e Paul Bartley pela recepção e orientação durante estágio em pesquisa no Instituto de Pesquisa Moredun na Escócia.
A todos os amigos e companheiros de laboratório Leonardo Mesquita, Raquel Gomes, Dennis Zanatto, Elena Cuevas, por toda ajuda em experimentos e bom humor durante todo esse tempo.
À professora Claudia Mori pelo suporte e ajuda no Laboratório de Modelos Animais.
À Professora Cristina Massoco pelo suporte no Laboratório de Farmacologia do VPT e também aos técnicos Herculano Pereira e Nicole Queiroz por toda a ajuda.
Ao Professor Bruno Cogliati e a técnica Tereza pela ajuda e suporte no LAPMOL no VPT.

Resumo

Costa, R. C. **Estudo de patogenicidade de duas cepas de *Neospora caninum* isoladas de caprinos e sua correlação genética com outras cepas por genotipagem de microssatélites.** [The Study of pathogenicity of two *Neospora caninum* strains isolated from goats and its genetic correlation with other strains by multiloccus microsatellite genotyping] 2020. 74p. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

O presente trabalho compreende o Estudo de diferentes cepas de *Neospora caninum* do Brasil e da Escócia quanto às suas características genéticas, avaliação de patogenicidade, virulência e possibilidade de isolamento. O primeiro capítulo, consiste de um artigo publicado no periódico internacional *Veterinary Parasitology* e descreve uma tentativa de isolamento de cepas do parasito utilizando tecido placentário de cabras naturalmente infectadas, em um bioensaio utilizando gerbis. Apesar dos gerbis serem descritos na literatura como sendo altamente susceptíveis à infecção por *N. caninum*, somente foi obtido sucesso na infecção de animais após imunossupressão com altas doses de metilpredinisolona. Animais imunossuprimidos apresentaram sinais clínicos de emagrecimento, pelos arrepiados e alterações de propriocepção como *Head tilt*. Análises histopatológicas revelaram reações inflamatórias no encéfalo, consistindo de infiltrados inflamatórios mononucleares multifocais associados à manguitos perivasculares e estruturas parasitárias semelhantes a cistos e taquizoítos, juntamente com áreas de necrose associadas à infiltrado inflamatório de macrófagos e reação de cicatrização em musculatura esquelética. Apesar do sucesso na infecção dos gerbis, não foi possível obter o isolamento do parasito em cultivo celular em uma primeira tentativa, o que levou à segunda parte do trabalho. O capítulo dois consiste de um artigo publicado no periódico intenracional *Experimental Parasitology.* Foram utilizados tecidos dos gerbis infectados descritos anteriormente e novas amostras de sistema nervoso central de caprinos naturalmente infectados em um bioensaio em camundongos C57BL6 Knockout para o gene Interferon gamma (camundongos knockout- KO). Os camundongos Knockout por serem incapazes de desenvolver uma resposta imune adequada ao *N. caninum*, permitiram a rápida replicação de taquizoítos principalmente em macrófagos peritoneais, os camundongos apresentaram acentuada perde de peso, letargia e ascite 15 a 30 dias após inoculação, quando foram eutanasiados e o líquido peritoneal utilizado para infecção de monocamadas de células VERO. A grande quantidade de taquizoítos no líquido peritoneal de camundongos KO permitiu uma rápida adaptação e crescimento dos mesmos em cultura de células, dando origem a dois isolados de cabras nomeados NC-Goat1 e NC-Goat2. Os dois isolados foram testados quanto à sua Patogenicidade em um modelo de infecção

experimental em camundongos BALB/c, amplamente utilizado na literatura. As cepas não apresentaram alta patogenicidade e virulência no modelo de infecção de camundongos, apesar das duas cepas terem sido associadas a doença clínica em caprinos, com abortos, natimortos e encefalite em caprinos jovens e adultos. O terceiro capítulo apresenta resultados de um estudo genético de genotipagem dos isolados NC-Goat1 e NC-Goat2, amostras clínicas de caprinos e bovinos do sul de Minas Gerais e amostras de bovinos da região de Dumfries e Galloway na Escócia. Utilizando a técnica de genotipagem por microssatélites, análises filogenéticas evidenciaram que as cepas de *N. caninum* presentes nos isolados e nas amostras clínicas de caprinos tinha relação próxima com os isolados brasileiros NC-SP1, NC-Bahia e NC-Goiás, enquanto que as amostras de bovinos de minas gerais não se correlacionaram proximamente às outras. Analisando as amostras provenientes de rebanhos escoceses, todos tenderam a se relacionar proximamente entre elas e com outras amostras escocesas descritas anteriormente na literatura. Análises adicionais utilizando marcadores microssatélites descritos pelo grupo de pesquisa do Instituto Moredun, evidenciaram genótipos mistos associados ao isolado padrão de *N. caninum* (NC-1), sugerindo que o isolado poderia ter sido composto por múltiplos genótipos ao isolamento e à medida que foi replicado em cultura celular convergiu em uma população mais homogênea de parasitos.

Palavras-chave: Apicomplexa, Abortos, Bioensaio, Microssatélites

Abstract

Costa, RC. **The Study of pathogenicity of two *Neospora caninum* strains isolated from goats and its genetic correlation with other strains by multiloccus microsatellite genotyping.** [Estudo de patogenicidade de duas cepas de *Neospora caninum* isoladas de caprinos e sua correlação genética com outras cepas por genotipagem de microssatélites]. 2020. 74p. Tese (Doutorado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

The present work comprises the study of different strains of Neospora caninum, from Brazil and Scotland, regarding their genetic characteristics, pathogenicity, virulence and attempts at isolation. The first chapter consists of an article published in the journal Veterinary Parasitology and describes an attempt to isolate parasite strains using placental tissue from naturally infected goats in a bioassay using gerbils. Although gerbils are described in the literature as highly susceptible to N. caninum infection, successful infection of animals has only been achieved after immunosuppression with high doses of methylpredinisolone. Immunosuppressed animals showed clinical signs of weight loss, fur and proprioception changes such as Head tilt. Histopathological analysis revealed inflammatory reactions in the brain consisting of multifocal mononuclear inflammatory infiltrates associated with perivascular cuffs, cyst-like and tachyzoite-like parasitic structures, along with areas of necrosis associated with macrophage inflammatory infiltrate and skeletal muscle scar formation. Despite the success in gerbil infection, it was not possible to obtain isolation of the parasite in cell culture in a first attempt, which led to the second part of the work. The chapter two consists of an article published in the journal Experimental Parasitology. Tissues from the previously described infected gerbils and new samples from the central nervous system of naturally infected goats were used in a bioassay using C57BL6 interferon gamma Knockout mouse (KO mice). Knockout mice, being unable to develop an adequate immune response to N. caninum, allowed the rapid replication of tachyzoites mainly in peritoneal macrophages, showing marked weight loss, lethargy and ascites 15 to 30 days after inoculation, when they were euthanized, and the peritoneal fluid used for infection of VERO cell monolayers. The large amount of tachyzoites in the peritoneal fluid of KO mice allowed their rapid adaptation and growth in cell culture, resulting in two goat isolates named NC-Goat1 and NC-Goat2. Both isolates were tested for pathogenicity in a widely used experimental BALB/c mouse infection model. The strains did not show high pathogenicity and virulence in the mouse infection model, although both strains were associated with clinical disease in goats, with abortions, stillbirths and encephalitis in young and adult goats. The third chapter presents the results of a genetic genotyping study of the isolates NC-Goat1 and NC-Goat2, clinical samples from goats and cattle from southern Minas Gerais

and samples from cattle from the Dumfries and Galloway region of Scotland. Using the microsatellite genotyping technique, phylogenetic analyzes showed that N. caninum strains present in the isolates and clinical samples of goats had a close relationship with the Brazilian isolates NC-SP1, NC-Bahia and NC-Goiás, while the samples from Minas Gerais cattle did not correlate closely to the others. Analysing samples from Scottish herds, all tended to relate closely to each other and to other Scottish samples previously described in the literature. Additional analyses using microsatellite markers described by the Moredun Institute research group showed mixed genotypes associated with the standard *N. caninum* isolate (NC-1), suggesting that the isolate could have been composed of multiple genotypes on isolation and as it was replicated in cell culture they converged to a more homogeneous population of parasites.

Keywords: Apicomplexa, Abortions, Bioassay, Encephalitis

Sumário

1- Introdução Geral

O protozoário do filo Apicomplexa Neospora caninum é conhecido mundialmente por ser o principal agente causador de abortos em rebanhos, principalmente de gado leiteiro (Dubey and Schares, 2011), causando apenas no Brasil perdas estimadas em U$51,7 milhões anualmente, em propriedades leiteiras, e U$101 milhões em propriedades de corte, sendo que as perdas anuais considerando 10 países no ano de 2013 chegaram a um valor estimado de U$1,298 bilhões (Reichel et al., 2013).

Além de bovinos a neosporose também afeta outras espécies de ruminantes domésticos e selvagens, como caprinos, ovinos bubalinos e cervídeos (Dubey and Schares, 2011) que atuam em seu ciclo como hospedeiros intermediários (HI). O cão doméstico e outros canídeos selvagens desempenham papel importante no ciclo do parasito, sendo hospedeiros definitivo (HD) (Dubey et al., 2017), eliminando oocistos infectantes pelas fezes após eventual consumo principalmente de placenta ou carne de animais infectados (Almeria et al., 2003; McAllister et al., 1998). É esperado que o parasito esteja presente em qualquer região onde seus hospedeiros intermediários e definitivos estejam presentes (Goodswen et al., 2013), atualmente, em todas as localidades onde pesquisas para identificação do agente foram conduzidas, o mesmo foi encontrado (Guido, 2017).

Desde sua descrição em 1988, vários estudos demonstram diferenças no comportamento do parasito em relação a sua patogenicidade e virulência, relacionados a diferentes isolados, em modelos de infecção in-vitro e in-vivo. (Dubey et al., 2017). Isolados viáveis de N. caninum já foram obtidos de bovinos, ovelhas, búfalos, cavalos, cervídeos e recentemente de caprinos, sendo esse último descrito por nosso grupo de estudos, com o isolamento de duas cepas provenientes de caprinos naturalmente infectados e nomeadas NC-Goat1 e NC-Goat2 (Costa et al., 2019). Apenas poucos desses isolados foram estudados com maiores detalhes contribuindo grandemente para o entendimento da neosporose em animais em consideração à imunogenicidade, patogenicidade, virulência e outras variáveis que diferenciam as cepas entre si (Dubey et al., 2017). A literatura descreve que isolado provenientes de animais que manifestavam sinais clínicos (abortos em bovinos ou doença neuromuscular em cães) possuem alto grau de patogenicidade e virulência, sendo os isolados NC-1 e NC-LIV (isolados de cães) e NC-SpainH7 (isolado de bovino) considerados os mais patogênicos em modelos de infecções experimentais (Dubey et al., 2017; Regidor-Cerrillo et al., 2008) . Por outro lado, os isolados de caprinos, apesar de

terem sido correlacionados várias vezes com episódios de abortos e transmissão congênita, possuíram baixa virulência em um modelo de infecção em camundongos, contradizendo o que era estabelecido anteriormente (Costa et al., 2019).

O genoma do N. caninum foi descrito em 2012 (isolado LIV-C, obtido de um cão), comprovando que o protozoário é diferente do Toxoplasma gondii, mas com um alto grau de ortologia com poucos rearranjos cromossomais entre eles. A pesar da disponibilidade de tecnologias que facilitam o sequenciamento genômico de organismos hoje em dia, essa técnica pode não ser acessível em todos os laboratórios. Marcadores microssatélites foram descritos para o N. caninum e apresentam alto grau de discriminação entre isolados já estabelecidos e cepas presentes em amostras clínicas de animais infectados pelo protozoário (Guido, 2017; Pedraza-Díaz et al., 2009). Pedraza-Díaz descreve 12 marcadores (marcadores “MS” - MS4, MS5, MS6a, MS6b, MS7, MS8, MS10, MS12, MS21) que combinados são capazes de discriminar genótipos entre si e relacioná-los a regiões geográficas onde são encontrados. Em um estudo realizado no Instituto de Pesquisa MOREDUN no reino Unido (Processo FAPESP 2018/16546-6) foi realizada a genotipagem dos isolados de cabras e de amostras clínicas de caprinos e bovinos da mesma região, utilizando a técnica de genotipagem por microssatélites, demonstrando que os isolados e as amostras clinicas possuíam múltiplas cepas do protozoário concomitantemente, sendo a primeira descrição de infecções mistas por cepas de N. caninum em uma população de animais. Utilizando os marcadores descritos por Guido et al., (2017) (Marcadores “MRI” - MRI2, MRI7, MRI14, MRI37, MRI41, MRI42) o estudo também sugere que isolados já estabelecidos, como a cepa NC-1, apresentavam múltiplos genótipos em culturas de baixa passagem em cultivo celular e convergiram para amostras mais homogêneas à medida que foram replicadas passando por várias gerações em cultivo celular, o que leva ao questionamento sobre a importância do estudo de infecções por múltiplos genótipos e como esses podem influenciar na patogenicidade e desenvolvimento da doença nos animais infectados.

Alguns estudos demonstram que animais expostos à uma infecção prévia, com cepas de baixa virulência, podem desenvolver proteção contra uma infecção subsequente por cepas de alta virulência (Rojo-Montejo et al., 2009), reforçando assim a importância do estudo de infecções mistas, já que essas podem mimetizar mais fidedignamente as infecções naturais. Infecções mistas podem estar relacionadas à introdução de novos genótipos de N. caninum em um rebanho por transmissão horizontal, a partir da ingestão de oocistos esporulados nas pastagens, eliminados em fezes de cães.

Os resultados desse trabalho demonstram a importância do estudo de novas cepas de *N. caninum* e cria precedentes para novos estudos que reavaliem os conhecimentos que temos sobre avaliação de patogenicidade de novas cepas e isolados.

Neospora caninum bioassay in gerbils using placental tissue of naturally infected goats

2- Neospora caninum bioassay in gerbils using placental tissues from naturally infected goats

This chapter presents an article published in the journal **Veterinary Parasitology.**

Abstract

Neospora caninum is one of the main agents that causes abortions in cattle worldwide. However, little is known about the pathogenesis of neosporosis in small ruminants, especially goats. Gerbils (*Meriones unguiculatus*) have been used as a model for neosporosis, and this species is highly susceptible to infection by bovine *N. caninum* strains. The present study aimed to evaluate the susceptibility of gerbils to a *N. caninum* strain from goats. The placentas were obtained from naturally infected goats, that presented with mild to severe lymphoplasmacytic and histiocytic infiltrate, foci of necrosis, calcification and protozoan-like structures. Immunosuppressed gerbils bioassayed with *N. caninum*-infected placental tissues showed severe neurological signs. Microscopic lesions in these gerbils were characterized by encephalitis, myocarditis, myositis and pancreatitis. These lesions were often associated with a small to moderate number of *N. caninum* tachyzoites, confirmed by immunohistochemistry and PCR. This is the first report showing that goat *N. caninum* strains could infect immunocompetent gerbils and cause severe lesions and clinical signs in immunosuppressed gerbils.

Keywords

Meriones unguiculatus, *Capra hircus*, Apicomplexan parasite, Encephalitis, Immunohistochemistry, PCR.

2.1- Introduction

Neospora caninum is an apicomplexan parasite that is known worldwide as an important cause of abortion, especially in dairy cattle (Dubey and Schares, 2011). There are few reports about the pathogenicity of *N. caninum* strains in goats, and to date, there are no reports of *N. caninum* isolated from goats (Porto et al., 2016). Few reports show that *N. caninum* can cause reproductive disorders and encephalitis in goats (Mesquita et al., 2013; Costa et al., 2014). Gerbils (*Meriones unguiculatus*) have been widely used as a model for neosporosis because this species is considered to be more susceptible to infection compared to inbred and outbred mouse strains (Ramamoorthy et al., 2005). To date, no studies have evaluated the pathogenicity of *N. caninum* from naturally infected

goats in gerbil models. Therefore, the aim of the present study was to evaluate the susceptibility of gerbils to infection by *N. caninum* goat strains.

2.2- Materials and methods

The *N. caninum* samples used in the present study were obtained from the placenta of three goats who had a history of previous abortions and congenital transmission of *N. caninum* to goat kids (Mesquita et al., 2013; Costa et al., 2014). These goats tested negative for agents that may cause abortions, such as *Toxoplasma gondii*, *Brucella* spp., *Coxiella burnetti* and *Chlamydophila abortus*. All animals were located in an experimental paddock, free of contact with dogs, and with periodic *N. caninum* IFAT testing in negative animals, to guarantee the absence of the possibility to a new horizontal infection by *N. caninum* oocysts from other routes as water and food. The placentas of these goats were collected immediately after parturition, and samples were collected for DNA extraction, histology and immunohistochemistry (IHC) to confirm the presence of *N. caninum*. Fresh placental samples were processed for inoculation of gerbils. Briefly, 100 grams of cotyledons were randomly collected from different areas of the placenta, and the cotyledonnary epithelium was aseptically debrided with a scalpel blade and digested with a 0.25% trypsin-EDTA solution for 45 minutes at 37°C. Then, the samples were filtered in a 70 µm cell strainer, washed twice with phosphate buffered saline, centrifuged at 1200 g for 10 minutes and suspended in RPMI 1640 medium to a final volume of 10 ml. The placental suspensions were intraperitoneally inoculated in six gerbils (0,5 ml for each gerbil) for each goat (case Nos. 1-18). Three gerbils from each group were immunosuppressed with three subcutaneous doses of 0.4 mg methylprednisolone at an interval of 7 days (case Nos. 4-6, 10-12 and 16-18). As controls, six gerbils were inoculated with the same volume of RPMI used for inoculation of experimental animals. Three control gerbils were immunosuppressed as previously described. The gerbils were evaluated daily and were euthanatized with an overdose of isoflurane on the 30th day post inoculation. All procedures in this work were approved by the Ethics Committee for animal use of the Universidade Federal de Lavras, under the protocol number 018/17.

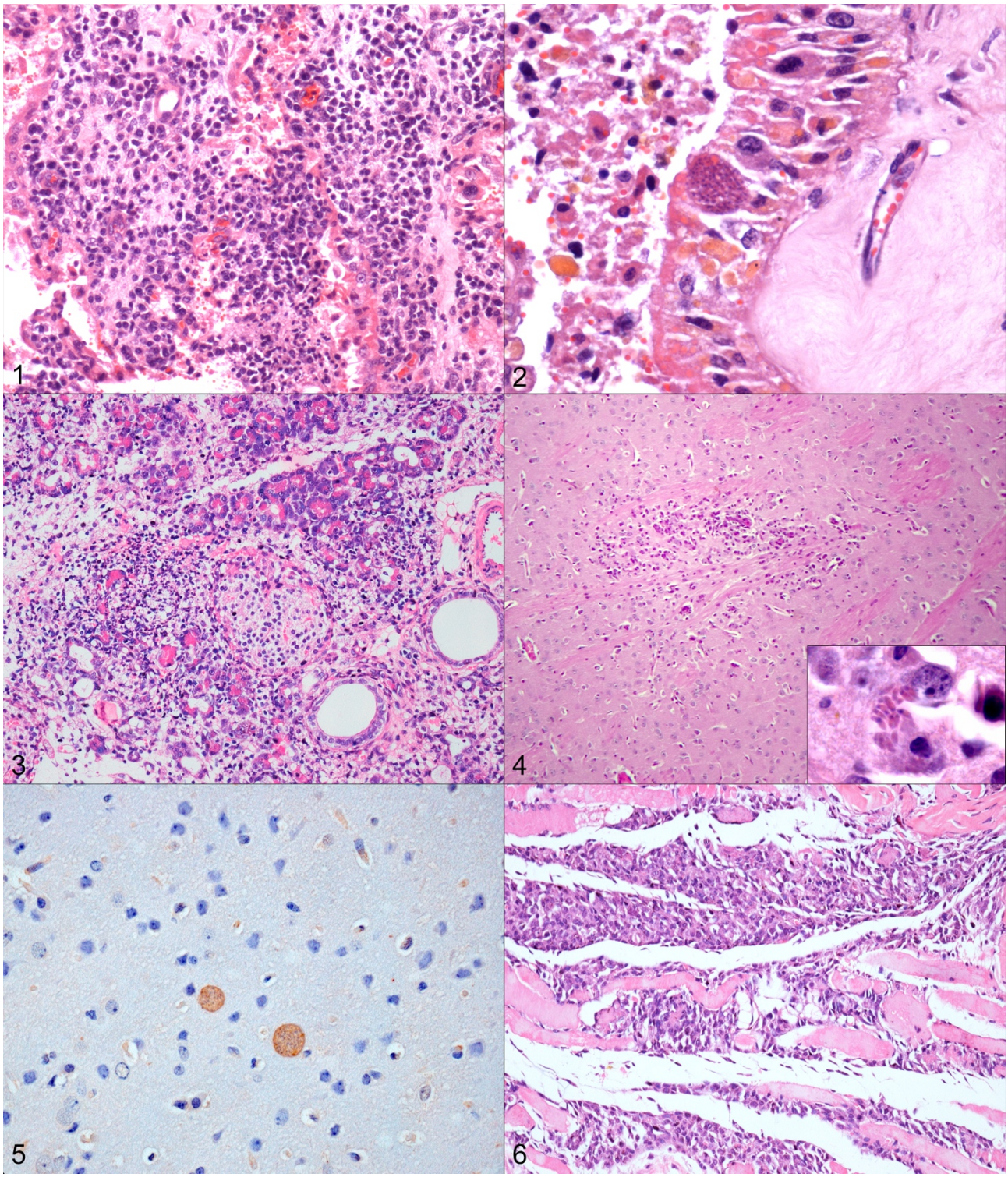
Tissue samples of brain, hearth, skeletal muscle, pancreas, kidney, liver, lung, spleen, eye, tongue, testicles, and mesenteric lymph nodes were collected in 10% buffered formalin for histology and IHC for *N. caninum.* IHC was performed following a standard protocol with a peroxidase method as previously described (Costa et al., 2014). In tissues from gerbils, a semi-quantitative analysis of IHC was performed, in which IHC was classified

as mild, moderate or severe. Mild IHC labeling was determined when rare small parasite structures resembling tachyzoites were observed within histological tissues. A moderate IHC labeling was used when numerous structures resembling tachyzoites and up to 5 aggregated parasites structures resembling cysts were observed in 5 high power fields (HPF, 400X), and severe IHC labeling was used when more than 5 cysts were observed in 5 HPFs. DNA from the placentas and gerbil tissues (brain, pancreas, liver, spleen, heart and skeletal muscle), were extracted with a commercial kit (Reliaprep gDNA Tissue Miniprep System - Promega), according to the manufacturer's instructions. Detection of *N. caninum* DNA was performed using primers for a specific region of the Nc5 gene as previously described (Collantes-Fernández et al., 2002). PCR was performed for detection of presence or absence of *N. caninum* NC5 gene. Serum samples from gerbils were collected one day before inoculation and at day of euthanasia, and from the goats at parturition, for the indirect fluorescent antibody test (IFAT) (Bandini et al., 2011; Mesquita et al., 2013). An initial serum dilution of 1:50 was used. Then, the slides were labeled with FITC conjugated Rabbit anti Gerbil IgG (ICCL) or caprine fluorescein-conjugated anti-IgG antibody (Sigma-Aldrich) both at 1:100 dilution. PCR for amplification of the MS10 microsatellite was performed according to Regidor-Cerrillo et al. (2006), and the product was sequenced by Sanger sequencing method.

2.3- Results and discussion

Goat 1 had an IFAT titer of 1:3200 at parturition, and both goats 2 and 3 had titers of 1:1600. Grossly, there was no lesions within the placentas. Microscopically, the placenta of goat 1 had a moderate to severe placentitis with multifocal infiltrates of a large number of macrophages and lymphocytes, and a lesser number of plasma cells within the stroma (Fig. 1), with multifocal areas of calcification and focal necrotic areas within the chorionic villi involving the trophoblastic cells. Protozoan-like structures were observed within the chorionic epithelium (Fig. 2) and stroma associated or not with inflammation. In the placentas of goats 2 and 3, a mild and multifocal lymphohistiocytic placentitis with few foci of calcification were observed. Structures resembling cysts and tachyzoites of *N. caninum* located mainly on the chorionic epithelium but also on the stroma of goat 1 and 2 were immunolabeled for *N. caninum*. Immunolabeled protozoan-like structures were not detected within the placenta of goat 3. The placentas from all goats (goats 1, 2 and 3) were positive for *N. caninum* NC5 gene.

The results about gerbils IFAT titer, clinical signs, PCR and IHC are summarized in Table 1.



Figures 1-2. Placenta of goats naturally infected with *Neospora caninum.* Figure 1. Placenta, goat 1. Severe inflammatory infiltrate of macrophages, lymphocytes, and plasma cells within the stroma. Hematoxylin and eosin (HE).
Figure 2. Placenta goat 1. Another area showing a large protozoan cyst-like structure within the trophoblastic epithelium, note the absence of inflammation in this area. HE. **Figures 3-6. Gerbils infected with *Neospora caninum* at 30 days post inoculation.**

Figure 3. Pancreas, gerbil No. 4. Histiocytic and lymphocytic pancreatitis with mild fibrosis. HE.
Figure 4. Brain, temporal cortex, gerbil No. 16. Focal area of gliosis. Insert: protozoan-like structures within the area of gliosis that are 2-3 µm in length with a unique central to slightly eccentric nucleus. HE.
Figure 5. Brain and temporal cortex, gerbil No. 5. Strong immunolabeling of *N. caninum* cyst-like structures. Immunohistochemistry for *N. caninum*.
Figure 6. Skeletal muscle, gerbil No. 4. Multifocal to coalescent, and severe histiocytic myositis. HE.

All gerbils inoculated with placental suspension from goat 1 (case Nos. 1-6) seroconverted at 30 days post inoculation (dpi), while 4 gerbils inoculated with placental suspension from goat 2 (case Nos. 7, 10-12), and two gerbils from goat 3 (case Nos. 16-17) seroconverted at 30 dpi. Two gerbils (4, 16) presented with significant clinical signs, such as a severe head tilt at 16 and 28 dpi, respectively, and there was apparently no loss of consciousness or coordination. Grossly, the pancreatic surface was slightly shrunken (case Nos. 4-6, 16). In all gerbils that seroconverted, *N. caninum* DNA was detected within the brain (case Nos. 1-7, 10-12, 16-17). *N. caninum* DNA was also detected within the pancreas, liver (case Nos. 4-7, 10-12, 16), heart and skeletal muscle (case Nos. 4-6) of *N. caninum*-infected gerbils. Histologically, the main affected organs of *N. caninum*-infected gerbils included the pancreas, brain, skeletal muscle and heart. The pancreatic architecture was partially effaced by a large number of macrophages and neutrophils as well as a small number of lymphocytes and plasma cells. There was also a multifocal necrosis and mild to moderate pancreatic fibrosis (Fig. 3) (case Nos. 4-7, 10-12 and 16-17). In the brains of *N. caninum*-infected gerbils (case Nos. 4, 5, 6, 10, 11, 12 and 16), there was a multifocal, mild to severe gliosis that was mainly in the telencephalon, thalamus, and brainstem (Fig. 4). Central nervous system (CNS) lesions were more prominent in gerbils that exhibited severe clinical signs (case Nos. 4 and 16). A few groups of tachyzoites-like structures (Fig. 4), as well as intraneuronal cysts-like structures labeled by *N. caninum* in IHC (Fig. 5), were observed associated or not with areas of gliosis. Only gerbils inoculated with placental suspension from goat 1 had myocardial and skeletal muscle lesions. Myocardial and skeletal muscle fibers were mildly to moderately expanded by a large number of macrophages with multifocal areas of muscle fiber degeneration and necrosis with dystrophic calcification (Fig. 6). Within the myocardium and skeletal muscle, there were multifocal to coalescing areas of fibrosis. Rare tachyzoite-like structures were immunolabeled for *N. caninum* within the heart and skeletal muscle.

The samples from three goats in this study presented the MS10 microsatellite allele $(ACT)_6(AGA)_{15}(TGA)_8$. The microsatellite MS10 alone have been

used to differentiate *N. caninum* strains (Reis et al., 2016; Peters et al., 2017). This allele, which was found in the *N. caninum* genome from the goats in the present study, was also found in a frequency between 20% and 30% in German and Scotland, and with a low frequency in Argentina and Spain (Regidor-Cerrillo et al., 2013). However, this allele was not previously described in *N. caninum* strains from Brazil.

In the present study, gerbils were successfully infected by *N. caninum* using placental tissue from naturally infected goats. These placental tissues had lesions, which were characterized by mild necrosis and calcification with moderate to severe lymphohistiocytic inflammatory infiltrate. In addition, goat 1 had a large number of parasite-like structures observed in histology and IHC. These placental lesions are similar to those described in experimental studies when the parasite was inoculated at mid-late gestational stage in cows (Regidor-Cerillo et al., 2014) and goats (Porto et al., 2016), which consisted of inflammatory infiltrates mainly composed by mononuclear cells and necrotic foci. Cattle inoculated at early gestational period with two *N. caninum* strains with different pathogenicity, NC-Spain7 (more pathogenic) and NC-Spain8 (less pathogenic), showed similar severe inflammatory infiltrate of mononuclear cells and rare neutrophils and presented a high rate of fetal loss (Regidor-Cerillo et al., 2014). In contrast, goats experimentally infected at early and mid-gestational periods with the NC-Spain7 strain showed more severe lesions consisting by moderate to extensive necrotic placentitis with mild non-purulent inflammatory infiltrate, and at a late gestational period, the necrotic lesions and inflammatory infiltrate were mild (Porto et al.,2016). Similarly, cattle inoculated at early and mid-gestational period with NC-1 strain showed severe placental necrosis and nonpurulent inflammatory infiltrate (Maley et al., 2003; Macaldowie et al., 2004).

The seroconversion of immunocompetent gerbils inoculated with goat *N. caninum* strains shows that the parasite burden inoculated through the placental samples was enough to cause infection. However, the initial parasite load inoculated in gerbils and the parasite stage could not be evaluated in this study, as fresh placental tissue was used. In contrast, other studies show that gerbils are a highly susceptible to *N. caninum* infection, using tachyzoites from NC-Kr2 (Kang et al., 2009) and Nc-1 (Ramamoorthy et al., 2005) strains, and oocysts from NC-Liv strain (Dubey et al., 2000). In contrast, Oliveira et al. (2017), using the same number of oocysts as Dubey et al. (2000), but from a different strain, reported that gerbils did not develop clinical neosporosis, demonstrating that the susceptibility of gerbils to neosporosis may be also related to the parasite strain.

2.4- Conclusion

The results from the present study show that gerbils were successfully infected with *N. caninum* using caprine placental tissues. However, immunosuppression was needed for the development of neosporosis. For the first time, the MS10 allele was characterized in a *N. caninum* strain derived from goats, and it shows that the strain used in the present study is distinct from those already described in Brazil. More studies are necessary to isolate and prove the pathogenicity and genetic identity of this strain of *N. caninum* from goats, contributing to the understanding of neosporosis in caprine species.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

We thanks to the Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) for a PhD grant (process number 2016/05010-2) and financial laboratorial support (process number 2016/24856-0). We would also like to acknowledge Fundação de Apoio à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the support on the field research of this work (process number CVZ-PPM 00763-16).

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The pathogenicity of two *Neospora caninum* goat strains in a BALB/c mouse model

3- The pathogenicity of two *Neospora caninum* goat strains in a BALB/c mouse model

This chapter presents results published in the journal **Experimental Parasitology**.

Abstract

Goats are frequently described as an intermediate host for the protozoan *Neospora caninum*, manifesting the disease mainly by recurrent abortions with placentitis and encephalitis in fetuses. Several reports of natural and experimental infections in cattle and mice show differences in the immune response, and the outcome of the infection can be variable depending on the species affected and by the behavior of the infective strain. This study describes for the first time two *Neospora caninum* strains isolated from naturally infected goats from the state of Minas Gerais, Brazil. One placenta and one brain from different goats were processed for a first bioassay in gerbils (*Meriones unguiculatus*). Subsequently, a second bioassay was performed by inoculating the processed brain samples from gerbils into Interferon gamma (IFN-γ) knockout mice (KO mice). Tachyzoites collected from the peritoneal fluid of the KO mice were inoculated into VERO cell monolayers, where they presented a very slow growth rate. The tachyzoites were also inoculated into BALB/c mice with a dose of 10^6 tachyzoites per animal. After a 5-week follow up, the animals infected with both of the strains developed a strong polarized Th1 response with increased serum and spleen gene expression levels of pro-inflammatory cytokines (mainly IFN-γ and TNF-α) in the first week. Tissue lesions were mild in the animals infected with both strains. Despite the strong immune response preventing an infection in the visceral organs, the parasite was able to reach the brain, causing progressive brain lesions from the second to fifth week post infection. The NC-goat1-infected mice presented with severe meningoencephalitis, but the NC-goat2-infected animals had considerable histological brain lesions only at week 5. Immunohistochemical analysis of the mouse brains revealed a different pattern of inflammatory cells compared to the naturally infected goats. A severe inflammatory infiltrate of $CD3^+$ T lymphocytes was found in the NC-goat1-infected mice. A more discrete infiltrate of $CD3^+$ T cells was found in the NC-goat2-infected animals. Additionally, IBA1 IHC revealed an intense microglial reaction and monocyte perivascular cuffs in the NC-goat1-infected animals and lower microglia/monocyte infiltrates in the NC-goat2-infected mice. This work contributes knowledge on the pathogenicity of new *Neospora caninum* strains in mice, comparable with other well-established mouse models of the

disease, and demonstrates the importance of studying goats as an intermediate host of this parasite.

Keywords: apicomplexa, encephalitis, gene expression, immunohistochemistry, flow cytometry, *Capra hircus*

3.1- Introduction

The apicomplexan protozoan *Neospora caninum* is considered a major abortifacient agent in ruminants worldwide (Dubey and Schares 2011), causing significant economic loss estimated at U.S. $1.298 billion annually in worldwide cattle production (Reichel et al. 2013). In addition to cattle, other animals are *N. caninum* intermediate hosts including goats, sheep, buffalos, small mammals, birds and even dogs (Dubey and Schares 2011). Since the description of the parasite in 1988, many studies show clinical and experimental results, both in vivo and in vitro, demonstrating different biological behaviors of the parasite, which suggests that different strains are involved in neosporosis with different virulence factors (Dubey et al. 2017). Viable *N. caninum* has been isolated from cattle, sheep, water buffalo, horses, white tailed deer and axis deer (Dubey et al. 2017), but to date, there is no report of isolation from goats (Campero et al. 2018). Different isolates have variable outcomes in experimental models, and the study of several isolates can largely contribute to the understanding of neosporosis regarding virulence, immunogenicity and other variables that are different among the strains (Dubey et al. 2017).

Clinical neosporosis manifests in the intermediate hosts as recurrent abortions, low pregnancy rates, neurological symptoms, and a low average birth weight; however, the infected animals may also be clinically healthy (Dubey et al. 2017). The disease in goats is manifested as abortions, stillbirths, clinically healthy but infected kids, and uninfected offspring even with a seropositive mother (Corbellini et al., 2001; Costa et al. 2014, Mesquita et al. 2013; Corbellini et al., 2001; Varaschin et al., 2012). Goats used as experimental models of infection show similar placental lesions as infected cattle (Porto et al. 2016) and naturally infected goats (Costa et al. 2018, Mesquita et al. 2018). While cattle are the most affected species in terms of economic loss (Reichel et al. 2013), experimental models in cattle can be very expensive, and not all laboratories have the adequate facilities to perform cattle research (Aguado-Martínez et al., 2017). Therefore, many infection models use rodents as an alternative to predict the pathogenicity of the strains, providing insight for the development of new treatments and vaccines (Aguado-Martínez et al., 2017; Innes et al., 2002; Mols-Vorstermans et al., 2013).

To date, there are no studies regarding the outcome of the *N. caninum* goat strains in experimental mouse models. Therefore, the aim of this study was to characterize the pathogenicity of two isolates of *N. caninum* from naturally infected goats from southern Minas Gerais, Brazil and to compare the findings with the characteristics of other isolates in a BALB/c mouse model, giving prospects on new studies regarding the pathogenicity of these two new strains.

3.2- Materials and methods

All of the animal procedures were performed under the approval of the ethical guidelines established by the Committee of Ethics in Animal Experimentation from the School of Veterinary Medicine at the University of São Paulo.

3.2.1 *N.* caninum isolation

To isolate *N. caninum*, samples of different tissues from two naturally infected goats were collected. Briefly, these tissues were processed and were first inoculated into gerbils. Next, the gerbil brains were collected, processed and intraperitoneally inoculated into C57BL/6 IFN-$\gamma^{-/-}$ mice. *N. caninum* tachyzoites were harvested from the mice's peritoneal fluid and then inoculated into VERO cell monolayers.

Goat samples: The parasite was isolated from placental samples and offspring of two naturally infected goats from Minas Gerais, Brazil. The first goat (Goat 1) gave birth to a clinically healthy kid and a stillbirth. The placenta (P1) and stillbirth brain (B1) were collected for isolation and histopathological analysis. The second goat (Goat 2) gave birth to two male goats that were euthanatized at 8 months of life via an anesthesia overdose with Thiopental, and their brains (B2 and B3) were collected aseptically. The placenta of Goat 2 was also collected postpartum for histological analysis.

Gerbil bioassay: All goat tissues underwent mechanical liquefaction in a sterile blender with PBS, followed by digestion with trypsin-EDTA at a final concentration of 0.5% at 37°C for 1 hour. The tissues were washed twice with PBS by centrifugation at 1500 g for 15 min, and the final product was inoculated in at least 3 gerbils for each sample. At 30 days post inoculation (dpi), the gerbils were tested by IFAT for anti *N. caninum* antibodies, the positive animals were euthanatized with an isoflurane overdose and the brains were collected for the mouse bioassay.

Infection of C57BL/6 IFN-$\gamma^{-/-}$ mice: Gerbil brains were processed as described previously for the goat tissues and inoculated into C57BL/6 IFN-$\gamma^{-/-}$ mice (KO mice). After 15–20 dpi, the tachyzoites were collected from the mouse peritoneal fluid and replicated by successive passages in the KO mice.

Inoculation in Vero cells: Vero cell monolayers were incubated for 2 hours with the KO mice peritoneal fluid containing macrophages and tachyzoites and subsequently replaced with fresh culture medium (MEM) maintained in a 37°C incubator with 5% CO_2. The cells were analyzed daily for the presence of tachyzoites, and the medium was changed every 2 or 3 days.

3.2.2 N. caninum infection and experimental design

Tachyzoites were harvested from the KO mice peritoneal fluid, passed through a 27 G needle for macrophage disruption and counted in a Neubauer chamber. The tachyzoite viability was determined by staining the dead cells with trypan blue. A dose of $1x10^6$ live tachyzoites was inoculated intraperitoneally in 30 BALB/c mice for each *N. caninum* isolate. As controls, 15 BALB/c mice were inoculated with 200 µl of a lysate of peritoneal macrophages collected from healthy KO mice, with an initial count of $5x10^5$ macrophages per ml. During the experimental infection, both of the strains were at the 3rd passage in the KO mice.

The *N. caninum*-infected BALB/c mice were followed for 5 weeks post inoculation. At each week, 5 mice from each inoculated group and 3 from the control group were euthanatized by isoflurane overdose, followed by terminal blood collection from the heart. Several tissues were collected for histopathology (including the brain, spleen, liver, pancreas, mesenteric lymph nodes, thymus, heart, skeletal muscle, lung, kidney and intestines), and the spleen was also collected for flow cytometry and gene expression analysis. The right frontal lobe from each brain was collected and stored at -20ºC for PCR analysis.

3.2.3- Histopathology and immunohistochemistry (IHC)

Samples of the brain, liver, pancreas, kidney, spleen, tongue, skeletal muscle, heart, and mesenteric lymph nodes were fixed in 10% formalin and routinely processed for hematoxylin and eosin (HE) staining. The brain lesions were graded as mild ($^+$), moderate ($^{++}$) and severe ($^{+++}$). Mild lesions were considered as focal areas of perivascular cuffs and meningeal infiltration with one layer of inflammatory cells. Moderate lesions were classified as focal areas with inflammatory infiltrate in the meninges and perivascular cuffs that have one to three layers of inflammatory cells. Severe lesions were classified as multifocal areas with inflammatory infiltrate in the meninges and perivascular cuffs that had more than three layers of inflammatory cells and with increased cellularity of neuroparenchyma (gliosis).

Immunohistochemistry (IHC) was performed for *N. caninum* using a polyclonal goat antibody (VMRD; Pullman, Washington, USA). The brain samples were also analyzed for the presence of inflammatory cells and MHC-II molecules using the antibodies CD3 (DakoAgilent, Santa Clara California, USA; 1:200); CD79-α (Dako, 1:100); Iba-1 (Merck Millipore, Darmstadt, Germany; 1:2500); MHC-II- IA/IE (Biolegend, San Diego, California, USA; 1:200); GFAP (Dako, 1:8000). The IHC followed a standard protocol as previously described by Costa et al. (2013). Briefly, all of the slides were submitted to antigen retrieval with a citrate buffer at pH 6.0 for 10 min in a pressure cooker. The sections were also submitted to peroxidase and protein blocking. Next, the slides were incubated with primary antibodies overnight at 4ºC. The antibody and antigen binding were visualized using the polymer system Envision Flex (Dako, Santa Clara, California, USA) and revealed with 3,3-diaminobenzidine (DAB; Dako).

3.2.4 Indirect fluorescent antibody test (IFAT)

The indirect immunofluorescence antibody test (IFAT) was performed using slides covered with *N. caninum* NC-1 tachyzoites produced in our lab and secondary antibodies to identify total IgG. The serum dilutions started at 1:50 for the mouse samples. A FITC-anti-mouse IgG (Sigma-Aldrich) was used as the secondary antibody, diluted to 1:100. For the infected gerbils, the serum was analyzed only for the presence or absence of anti-*N. caninum* IgG, with a serum dilution of 1:50 and a commercial FITC-conjugated rabbit anti-gerbil IgG (ICL, Lake Oswego, Oregon, USA) diluted to 1:100.

3.2.5 PCR

Samples from the right frontal lobe from all of the mice were collected and frozen at -20ºC for PCR. The DNA was extracted using a commercial kit (Reliaprep gDNA tissue Miniprep System, Promega) according to manufacturer's instructions. PCR was performed using the PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 100 ng of DNA and 5 mM of each forward and reverse primers for the *N. caninum* NC5 sequence as described by Collantes-Fernández et al. (2002). The reactions were performed in duplicate in a *StepOne™ Real-Time PCR System, version 2.2* (Applied Biosystems) for the presence or absence of the NC5 sequence.

3.2.6 Gene expression of inflammatory mediators

After necropsy, the spleens from the *N. caninum*-infected and control mice were immediately frozen in liquid nitrogen. These samples were macerated in liquid nitrogen, and 30 mg were submitted to RNA extraction using the RNAspin Mini RNA isolation kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The total extracted RNA was quantified, and reverse transcription was performed using the

Superscript II Reverse transcriptase (Invitrogen, Life Technologies). The gene expression analysis was performed in triplicate in a StepOne Plus System (Applied Biosystems) using the following *TaqMan* probes for several cytokines (IFN-γ, IL-12p40, IL-6, TNF-α), the chemokine CCL5, the toll-like receptor (TLR) 3, and iNOS. The reactions for GAPDH and rRNA 18s were performed for endogenous controls. Each reaction consisted of 10 µL of TaqMan Universal master mix 2x (Applied Biosystems), 100 ng of cDNA, 1 µL of TaqMan Probe and ultra-pure water for a final volume of 20 µL. The cycling consisted of UDG activation at 50°C for a 2 min initial denaturation at 95°C for 10 min, and 40 cycles of 95°C for 1 min and 60°C for 1 min for annealing and extension with fluorescence capture.

3.2.7 Measurement of inflammatory mediators

The serum level of the inflammatory mediators was measured in all 60 *N. caninum*-infected and 15 control mice. For this measurement, a BD cytometric bead array (CBA; Beckinton Dickinson, Franklin Lakes, New Jersey, California, USA) and mouse Th1/Th2/Th17 cytokine kit (Beckton Dickinson) was used following the manufacturer's protocol. Additionally, the cytokines and chemokines IFN-γ, IL-1α IL-1β, IL6, IL-12p40, IL-12p70, IP-10, MCP-1, MIP-1a, MIP-1b, MIP-2, MIG, RANTES and TNF-α were measured in serum samples from 3 mice from each group using a Luminex assay, which is a multiplex bead immunoassay with a custom Milliplex MAP mouse cytokine/chemokine magnetic bead panel (Millipore, Darmstadt, Germany). The Luminex assay was performed and analyzed according to the manufacturer's protocol.

3.2.8 Flow cytometry

Spleen cells from the *N. caninum*-infected and control mice were mechanically dissociated by compression with two glass slides, followed by gentle circular movements and PBS washes. The cells were then collected in 10% DMSO, 40% fetal calf serum and 50% RPMI and stored overnight at -80°C until they were transferred to liquid nitrogen. To perform flow cytometry, the cell vials were thawed at 37°C and washed with fresh RPMI. A total of $1x10^6$ viable cells per sample were used for analysis. The cells were then incubated with CD16/CD32 Fc-block (clone 93, Biolegend) and diluted to 1:20 for 30 min at 4°C. After the appropriate washing steps, the cells were incubated with two different antibody panels as follows: 1) Alexa Fluor 488 anti-mouse CD45 (Clone 30-F11, Biolegend, San Diego, California, USA), APC anti-mouse F4/80 (BM8), PE anti-mouse/human CD11b (M1/70), and Percp anti-mouse IA-IE (M5/114.15.2); and 2) FITC anti-mouse CD3 (biolegend), Percp anti-mouse CD4 (GK1.5), PE anti-mouse CD8 (53-6.7) and APC anti-mouse CD19 (1D3/CD19). The samples were analyzed in a Beckton Dickinson FACSAria, and a total of $1x10^5$ events

were collected for each sample. The analysis was performed with Kaluza Analysis software (Beckman Coulter, Brea, California, USA).

3.2.9 Statistical analysis

The statistical analysis for the inflammatory mediator and flow cytometry data was based on multiple T tests. The relative quantitative gene expression was calculated by the comparative method as described by Steibel et al. 2009.

3.3. Results

3.3.1 Goat sample analysis

Brain HE sections from the aborted fetus of Goat 1 had multifocal mild encephalitis with gliosis and perivascular cuffs composed of mononuclear cells. Multifocal mononuclear inflammatory infiltrates were observed in the placenta of Goat 1. Fewer placental lesions occurred in Goat 2 compared to Goat 1, with multifocal discrete infiltrates of mononuclear cells in the placental stroma under the trophoblast cell layer. The two male goats (Goat 2 offspring) presented with moderate encephalitis with gliosis and perivascular cuffs.

3.3.2 N. caninum isolation

Gerbil infection: At 30 dpi, the gerbils infected with the B1, B2, B3 and P1 samples that seroconverted were euthanatized, and the brain was processed for inoculation into the KO mice. The gerbils did not present any clinical sigs or histopathological lesions in any organs.

KO mice infection: The KO mice inoculated with the brain samples from the gerbils that were inoculated with the B1 and P1 goat samples developed severe peritonitis within 15 dpi. The other KO mice inoculated with the gerbil brains infected with the B2 and B3 goat samples developed peritonitis and showed clinical signs at 20 dpi. Subsequent infections were made in the KO mice using $1x10^5$ tachyzoites from a peritoneal wash. After the second passage, all of the KO mice developed clinical signs of weight loss, ruffled hair, and tremors, followed by death at approximately 8–9 dpi. The animals were euthanatized before being unable to walk and eat to avoid animal suffering.

Infection of cell cultures: The tachyzoites were visible in the VERO cells on the day following the inoculation. The parasite growth was very slow during the first few weeks of culture, and the tachyzoite recovery from the culture was insufficient to inoculate all of the BALB/c mice (data not shown).

3.3.3 The outcome of the N. caninum infection in the BALB/c mice

The *N. caninum*-infected mice did not present any clinical signs during the 5 weeks of follow up. At necropsy, a marked splenomegaly was visualized in all of the mice infected with both of the *N. caninum* strains, but only at week 1 postinoculation. At week 1, the main

microscopic lesion was characterized by a marked splenic white pulp hyperplasia and mild to moderate peritonitis with mononuclear inflammatory infiltrates within the omentum, mesenteries and serosa of the peritoneal organs. In the liver of the *N. caninum*-infected mice, the mild periportal inflammatory mononuclear infiltrates were slightly more prominent in the NC-goat1-infected mice **(Figure 1a)**. In the pancreas, there was moderate interstitial inflammatory mononuclear infiltrates **(Figure 1b)**. Between weeks 2 to 5, microscopic lesions were only detected within the brain.

The intensity and distribution of the lesions in the central nervous system (CNS) are presented in **Table 1**. In the NC-goat1-infected mice, the brain lesions were first visualized at week 2 and progressed until week 5. The brain lesions consisted of multifocal areas of meningitis and encephalitis with gliosis and perivascular cuffs **(Figure 1c)**. The cerebellum, cerebellar peduncles, and medulla were the most affected areas. In one infected mouse at week 5, a tissue cyst was visualized in an HE section **(Figure 1d).** IHC revealed multiple tissue cysts **(Figure 1e)** and tachyzoite-like structures **(Figure 1f)** associated with areas of inflammation and gliosis in the brains of the NC-goat1-infected mice at week 5. Parasite structures were not visualized in the tissues from the NC-goat2-infected mice. Only two NC-goat2-infected mice had histological lesions in the brain visible by HE sections at week 5 that were characterized by focal areas of mild perivascular cuffs and discrete gliosis.

Despite the absence of histological lesions in the CNS of the NC-goat2-infected mice until week 5, the IHC revealed multiple discrete $CD3^+$ cell infiltration at week 2 **(Figure 1g),** with an increase in the number of $CD3^+$ cells at subsequent weeks (**Figure 1h)**. In the NC-goat1-infected animals, moderate to severe $CD3^+$ cell infiltrates were visualized in the brain **(Figure 1i)**, consistent with areas of microglial hypertrophy and monocyte infiltration **(Figures 1j and 1k)**. In contrast, the NC-goat2-infected mice had a lower microglial reaction **(Figure 1l)**. CD79-α^+ cells, and cells expressing MHC-II were rare in the NC-goat 1-infected mice **(Figures 1m, 1n).** MHC-II was also moderately expressed by the endothelial cells associated with the inflammatory areas in the NC-goat1-infected mice. An astrocytic reaction was evident in the IHC at week 3 in the NC-goat1-infected mice but was not prominent (**Figure 1o)**. The NC-goat2-infected mice had markedly lower MHC-II expression in the endothelial cells, the CD79-α^+ cells were absent, and the astrocytic reaction was low. **Table 2** represents the lesion intensity according to the average number of marked cells by IHC within the areas of the encephalic lesions. The **Figure 2** presents the total IgG titers anti-*N. caninum* for the infected mice following 5 weeks of infection.

All of the brain samples from all of the infected groups were positive for the *N. caninum* NC5 gene, starting at week 2.

3.3.4. Gene expression

The gene expression analysis showed that IFN-γ and TNF-α were upregulated in the spleen of the mice infected with both *N. caninum* goat strains at week 1 (**Figure 3**). Between weeks 2 to 5, there was no difference in the IFN-γ, TNF-α, CCL5, IL12p40, iNOS and TLR3 expression in the spleen of the N. caninum-infected mice when compared to the controls.

3.3.5 Cytokine/chemokine serum levels

At week 1, all of the cytokines, except for IFN- γ and TNF-α, were below the detection level of the CBA kit (data not shown), showing a polarized Th1 response against the infection. The complementary analysis with the Luminex assay revealed significantly greater levels of TNF-α and IFN-γ ($p<0,05$) in the mice infected with both of the *N. caninum* strains at 7 dpi. When compared to the control mice (**Figure 4**), the levels of MIG, MCP1, and IP10 were increased in the mice infected with both of the *N. caninum* strains at weeks 1 and 2 (**Figure 4**). At week 2, the levels of MIG, MCP1, and IP10 were greater in the NC-goat2-infected mice when compared to the mice infected with NC-goat1. All of the other cytokine/chemokine levels between weeks 3 to 5 were not significantly different than the control mice.

3.3.6 Flow cytometry

The main response observed in the spleen of the *N. caninum*-infected mice was increased MHC-II expression by $CD45^+F4/80^+$ cells (macrophages) ($P<0,05$) **(Figure 5)**. At week 1, all of the infected mice had a significant increase in the number of $CD45^+CD11b^+$ cells ($p<0,05$) (Figure 18). There was no significant change in the proportions of $CD4^+$, $CD8^+$ and $CD19^+$ cells in the spleen of the *N. caninum*-infected mice when compared to the control mice during the 5 weeks evaluated **(Figure 18)**.

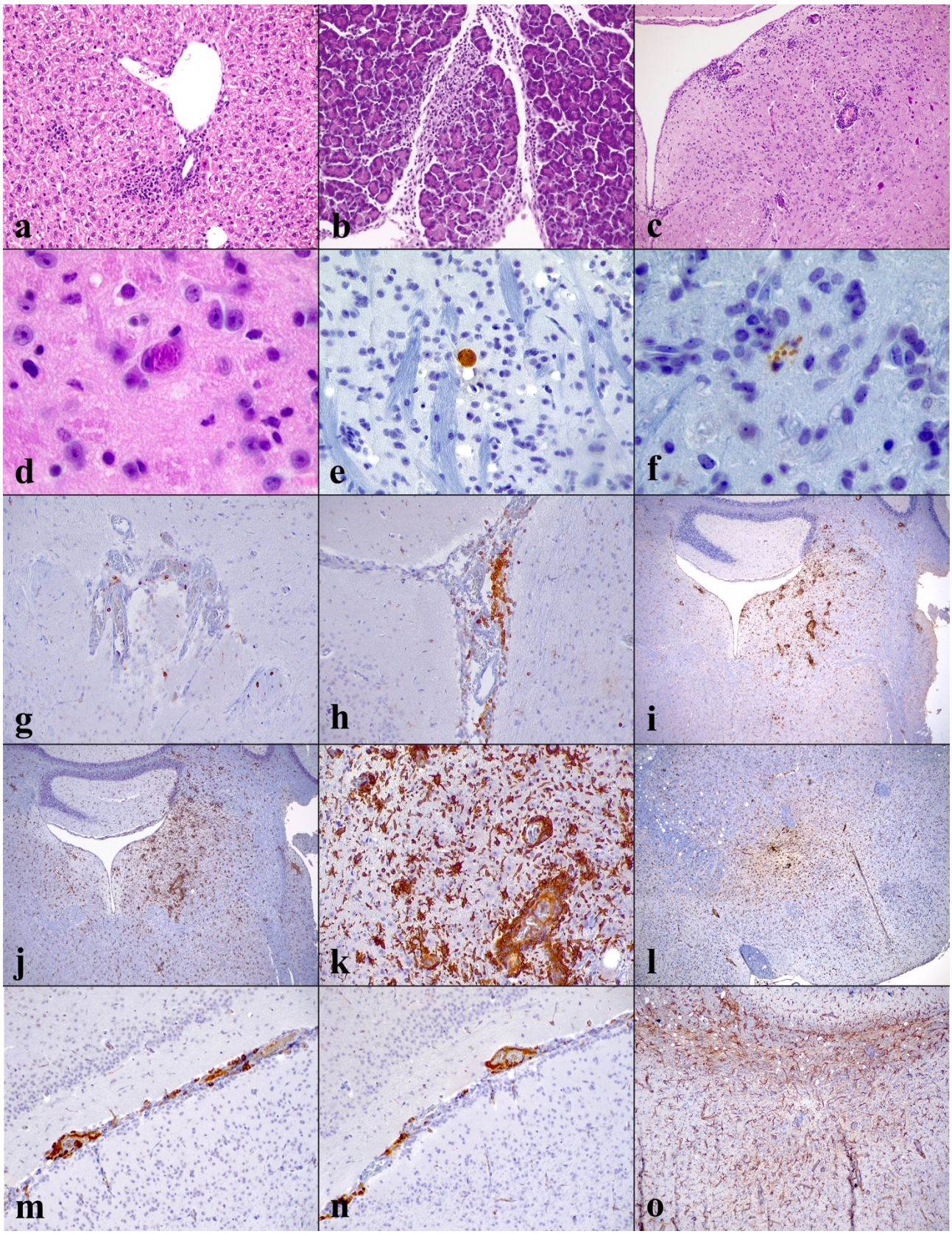

Figure panel 1 a-o: a- Liver, NC-goat1-infected mice, week 1 post inoculation, mild periportal inflammatory infiltration. (HE 400x). **b-** Pancreas, NC-goat2-infected mice, week 1, moderate interstitial inflammatory infiltrate. (HE 400x). **c-** Brain, NC-goat1-infected mice, week 2, the medulla and cerebellar peduncle with a severe inflammatory reaction with

perivascular cuffs and increased local cellularity of the neuroparenchyma (gliosis). (HE 200x). **d-** Brain, NC-goat1-infected mice, week 5, an intraneuronal *Neospora caninum* tissue cyst. (HE 1000x). **e-** Brain, NC-goat1-infected mice, week 5, intense immunolabeling of an *N. caninum* tissue cyst. (IHC anti-*N. caninum* 400x). **f-** Brain, NC-goat1-infected mice, week 5, immunolabeling of *N. caninum* cyst-like structures. (IHC anti-*N. caninum* 1000x). **g-** Brain, NC-goat2-infected animal, week 2, discrete $CD3^+$ cell perivascular infiltrates. (IHC anti-CD3, 200x). **h-** Brain, NC-goat2-infected mice, week 3, moderate $CD3^+$ cell perivascular infiltration. (IHC anti-CD3, 200x). **i-** Brain, NC-goat1-infected animal, week 2, severe $CD3^+$ cell infiltration. (IHC anti-CD3, 100x). **j-** Brain, NC-goat1-infected mice, week 2, a focally extensive area of microglial hypertrophy and monocyte infiltration. (IHC anti-IBA1, 100x). **k-** A high power field image of Figure **j**. Note the ramified microglial structures and round perivascular monocytes. (IHC anti-IBA1, 400x). **l-** Brain, NC-goat2-infected mice, week 5, a lower focal microglial reaction compared to the NC-goat1-infected animals. (IHC anti-IBA1, 100x). **m-** Brain, NC-goat1-infected mice, week 5, low CD79-α^+ cell perivascular infiltration. (IHC anti-CD79α, 400x). **n-** Brain, NC-goat1-infected mice, week 5, same area as Figure 13, MHC-II immunolabeling of perivascular and endothelial cells. (IHC anti-IA/IE, 400x). **o-** Brain, NC-goat1-infected mice, week 5, a moderate astrocytic reaction in an area of gliosis. (IHC anti-GFAP, 400x).

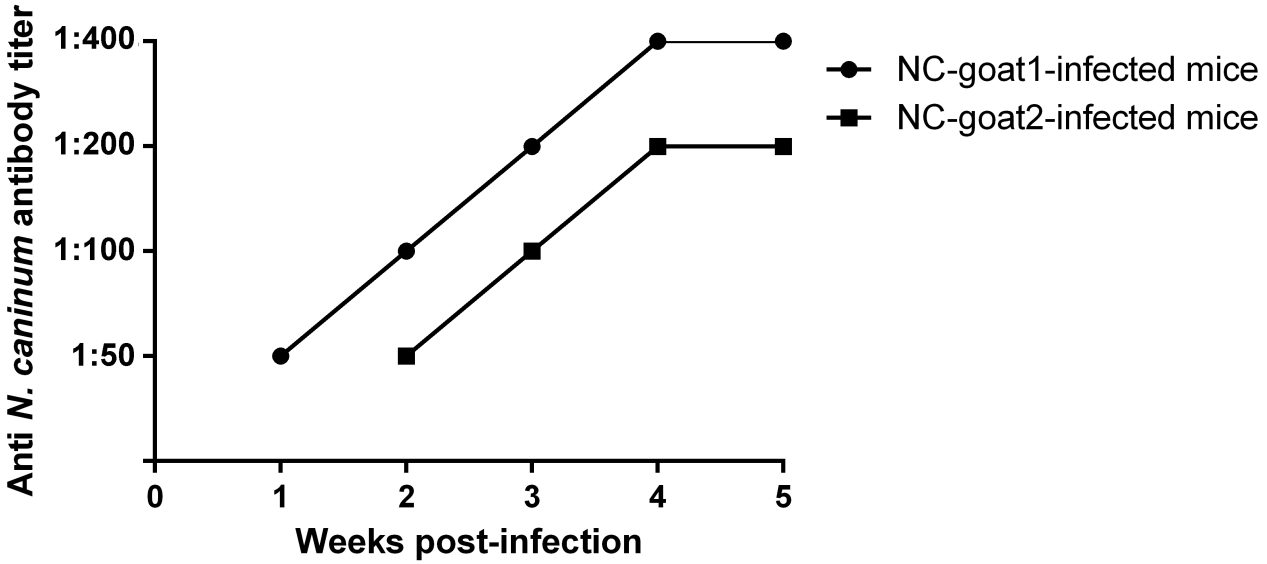

Figure 2: Anti-*Neospora caninum* total IgG titters of mice infected with strains NC-goat1 and NC-goat2, following 5 weeks of infection.

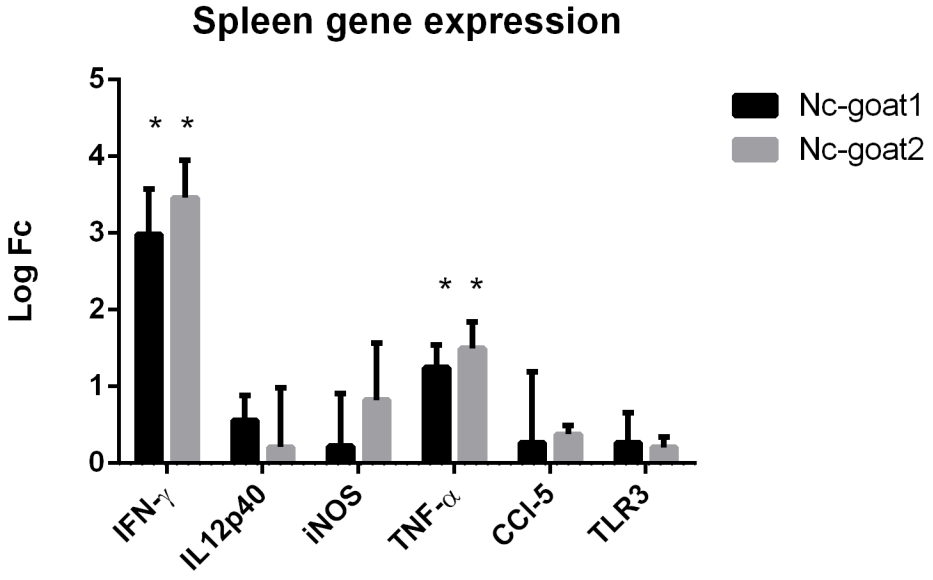

Figure 3: Gene expression of cytokines and chemokines IFN-γ, IL12p40, TNF-α, CCl5 and toll-like receptor 3 and the molecule iNOS in the spleen of mice infected with NC-goat1 and NC-goat2 strains at 1 week post inoculation. The data are expressed as the fold change (FC) estimates (log_2(FC)). The asterisks indicate a statistical significance (* $p < 0.05$).

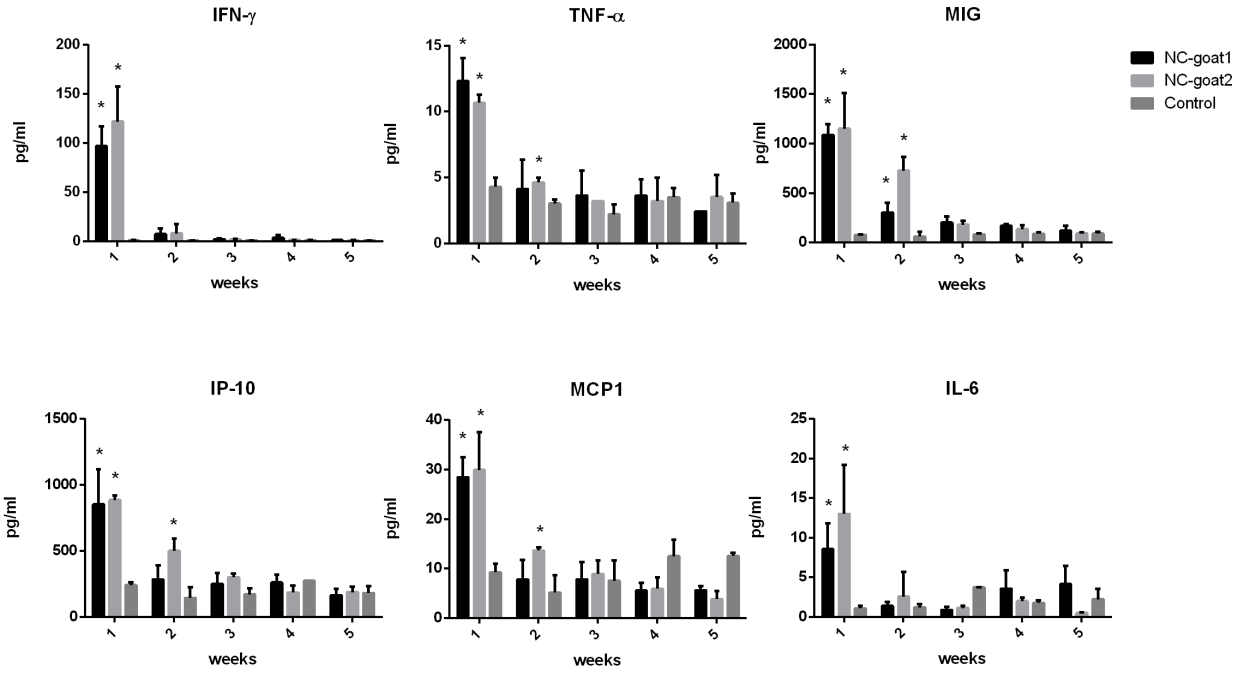

Figure 4: Serum levels of cytokines and chemokines IFN-γ, TNF-α, MIG, IP-10, MCP1 and IL-6 in NC-goat1 and NC-goat2-infected mice at 1 to 5 weeks post inoculation. The asterisks indicate statistical significance (* $p < 0.05$).

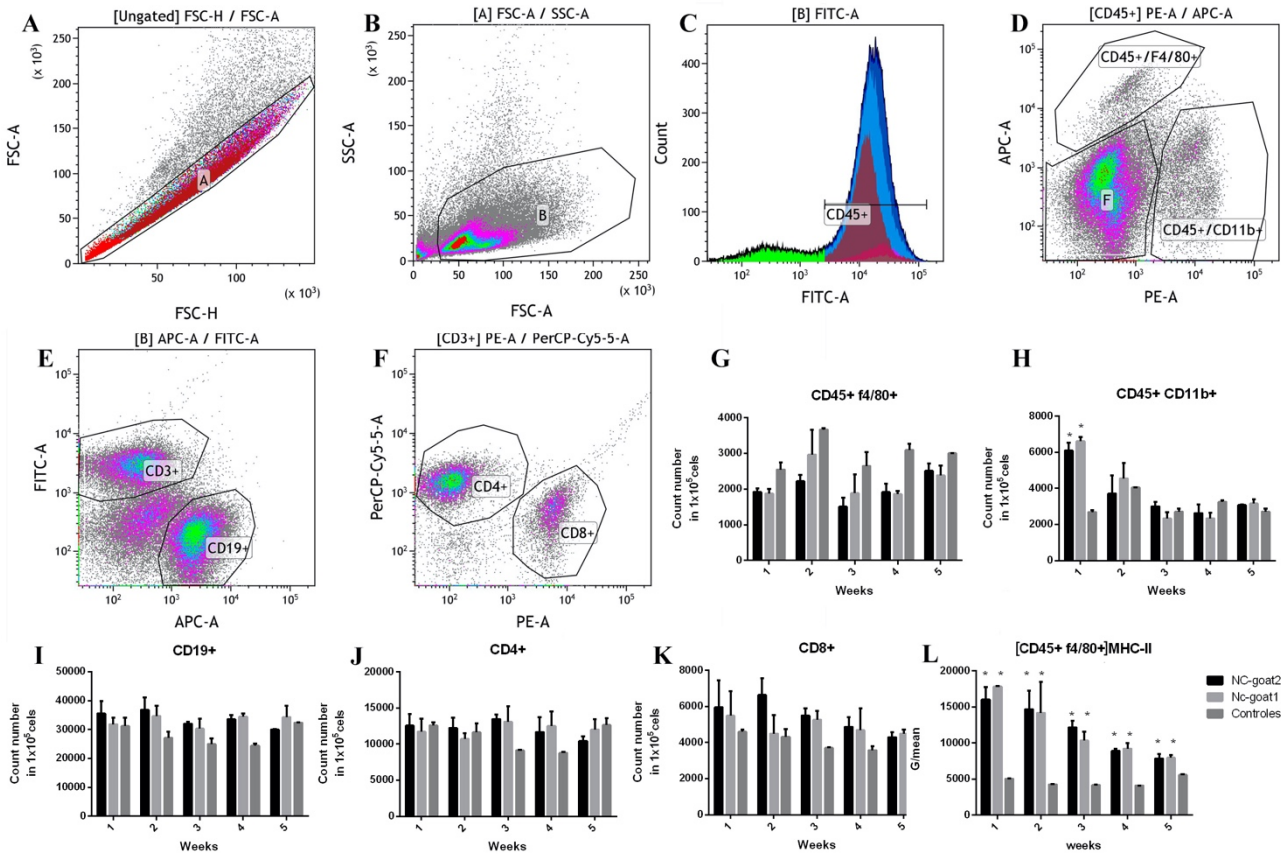

Figure 5: Flow cytometry analysis of spleen cells from the mice infected with the NC-goat1 and NC-goat2 strains. **A- Panel 1**- FSC-a/FSC-h exclusion of doublets. B- SSC-a / FSC-a, selection of leukocytes and exclusion of debris. C- Selection of leukocytes $CD45^+$. D- Phenotyping of $CD45^+$ cells as $CD45/F480^+$ (macrophages) and $CD45/CD11b^+$ (a mixed population of neutrophils, eosinophils, cDCs, pDCs, and NK cells). **E- Panel 2**- phenotyping of lymphocyte populations as T lymphocytes ($CD3^+$) and B lymphocytes ($CD19^+$). **F-** Phenotyping of $CD3^+$ cells as $CD3^+CD4^+$ (T helper) or $CD3^+CD8^+$ (T cytotoxic). **G, H, I, J, K-** Cell counts for NC-goat1 and NC-goat2 infected mice-cells at 5 weeks post inoculation. **L-** The geometric mean of the MHC-II cellular expression on the $CD45^+F4/80^+$ cells. The asterisks indicate statistical significance (* $p < 0.05$).

3.4. Discussion

The present study demonstrates the isolation of two *N. caninum* strains from naturally infected goats (NC-goat1 and NC-goat2) in both Vero cells and IFN-γ KO mice. We have previously demonstrated the pathogenicity of these strains in goats and gerbils (Costa et al., 2014, Costa et al. 2018, Mesquita et al., 2013). The vast amount of tachyzoites in C57BL/6 IFN-γ KO mice ascites fluid facilitated the infection and growth of tachyzoites in VERO cells. The growth rate was slow for the first passage and gradually increased in subsequent passages, similar to a previous report by Dubey et al. (1998) for the NC-4 and NC-5 strains. Several attempts were made to isolate the parasite in cell culture using infected gerbil brains.

However, there was no tachyzoite growth in the Vero cells during these attempts (Costa et al., 2018).

In the brain HE sections, the NC-goat1- and NC-goat2-infected mice presented moderate and mild multifocal nonsuppurative meningoencephalitis, respectively, with gliosis and mononuclear perivascular cuffs. Compared to the other BALB/c infection models, the mice infected with our goat strains (NC-goat1 and NC-goat2) did not developed the multifocal areas of necrosis in the brain that is usually demonstrated in experimental infections with NC-1 (Bartley et al. 2009, Long, et al. 1998), NC-spain7 (Pereira García-Melo et al. 2010), NC-Liverpool and NC-SweB1 (Atkinson et al. 1999) and NC-Nowra (Miller et al. 2002). In most of these cases, the necrotic areas were accompanied by macrophage infiltrates and gitter cells. The main inflammatory cell present in the NC-goat1- and NC-goat2-infected mice was $CD3^+$ T lymphocytes at week 2, followed by a microglial reaction and monocyte infiltration in subsequent weeks. Evaluations of the virulence of the *N. caninum* strains are largely performed in BALB/c mouse models (Dubey et al. 2017), but the lack of a standard model may make comparisons among the strains difficult (Innes et al. 2002). Not all of the studies on *N. caninum* infections in mice describe the histological lesions in the brain, and most studies using other *Neospora* strains classify the inflammatory infiltrate only as mononuclear or macrophage infiltrates, sometimes accompanied by gliosis but without phenotyping the cellular infiltrates (Atkinson et al. 1999, Bartley et al. 2009, Baszler et al., 1999, Lindsay et al., 1995, Long et al., 1998). Little is known about T cell participation in *N. caninum* encephalitis. Numerous T lymphocytes usually infiltrate the neuroparenchyma following viral infections (Hosking and Lane, 2010). The presence of T cells, especially CD8+, in the brain lesions of mice infected with *Toxoplasma gondii* correlates with an efficient immune response and parasite clearance in the CNS (Ochiai et al., 2015; Ochiai et al., 2016; Strack et al., 2002). The cellular pattern in the brain lesions of the NC-goat1- and NC-goat2-infected mice also differ from those observed in the CNS of naturally infected aborted fetuses and adult male goats, which predominantly had macrophages and microglial reactions accompanied by astrocytosis (Costa et al. 2014). The phenotyping of inflammatory cells in the CNS of mice infected with other *N. caninum* strains could elucidate the participation of these cells during *Neospora* encephalitis.

The mice infected with NC-goat1 presented brain tissue cysts and tachyzoite-like structures, but they were only visible at week 5 in 4 of the 6 animals. The tissue cysts are usually difficult to visualize in histological sections of the infected mouse brain, especially in BALB/c mice (Dubey et al. 1998, McGuire et al., 1997). The brain lesions in mice infected

with NC-1 (Lindsay et al. 1995, Long et al. 1998), NC-Liverpool and NC-SweB1 (Atkinson et al. 1999) are usually accompanied by the presence of tachyzoites associated with the inflammatory areas. The cysts and tachyzoites from the NC-goat1-infected mice were not always accompanied by inflammatory cells.

The systemic immune response to infection with the NC-goat1 and NC-goat2 strains in mice were evident during the first week, with high levels of IFN-γ and TNF-α in the serum and respective mRNA expression in the spleen. A strong polarized Th1 response with low IL-4, as expected in primo infections (Nishikawa et al., 2001), efficiently controlled the parasite infection in the visceral organs but could not prevent the spread and progression of the disease to the brain. No significant differences in the immune response were observed between the strains regarding the serum cytokine profiles. Neosporosis in experimentally infected mice occurs in the following two stages: 1) an early stage that produces systemic pathological responses followed by parasite clearance and 2) a chronic phase restricted to the brain (García-Melo et al. 2009). Khan et al. (1997) shows the greatest increase in the cytokine expression profiles occurring 6 h post infection, with high expression profiles continuing until weeks 1 and 2, but at an expression 10 times lower than the first peak 6 hours after inoculation. Significant changes in the cytokine expression profiles were observed mainly with in vitro stimulation of splenocytes from infected animals (Bartley et al. 2009, Baszler et al. 1999). These experiments suggest that the first hours and days of inoculation can be critical to determine the outcome of the disease systemically, but the immune response in the brain could be highly regulated by resident cells to avoid neuronal damage and is somewhat independent of the systemic response (Hosking and Lane, 2010; Klein and Hunter, 2017).

Flow cytometry analysis revealed increased MHC-II expression in $CD45^{+}F4/80^{+}$ cells from the spleen of our mice during all of the experimental weeks. The phenotype of these cells includes classical macrophages of the spleen (Rose et al., 2012), showing that they responded efficiently against the infection under a polarized Th1 response, and as expected, they were also sustained by the increased IFN-γ serum levels and the mRNA levels in the spleen. During the week of the increased proportion of $CD45^{+}CD11b^{+}$ cells, the phenotype included classical dendritic cells (cDCs) and plasmocytoid dendritic cells (pDCs) but also NK cells, neutrophils and eosinophils (Rose et al., 2012). Subclassifying the phenotype of these cells was not possible with the antibody panel used, but the *N. caninum* infection is characterized by an early response of cDCs and pDCs that are also correlated with the spread of the parasite to other organs and the response of the NK cells being early

producers of IL-12 (Teixeira et al., 2010). There was no significant change in the proportion of T and B cells in the spleen, but the mononuclear infiltrates in other organs, especially the brain, show activation of the T cell mediated immune response. Correia et al. (2013) show increased T $CD4^+$ and $CD8^+$ cells in the lymphoid organs of orally infected mice during the first hours following inoculation. In our work, as the spleen is not a target organ for the parasite, these cells could have migrated to other affected organs, especially the brain.

The lower IgG titers in the NC-goat2-infected mice could not be explained, as there were no significant changes in the cytokine profiles, or the cellular responses of the animals infected with both of the strains. We assume that the NC-goat2 had a lower replication rate during the mouse infection. Additionally, when observing the tachyzoite growth in cell culture, the NC-goat2 consistently took longer than the NC-goat1 to destroy the cell monolayer (data not shown). Alternatively, Bartley et al. (2009) show that the mice infected with attenuated strains produced more IgG titers than that challenged by a virulent one, which was explained by a greater IL-10 and IFN-γ production that could control the infection more efficiently. The IL-10 levels were not measured in the present work.

The mice infected with both of the NC-goat1 and NC-goat2 strains did not presented any clinical signs during the 5 weeks follow-up, which is consistent with the previously reported low virulent strain NC-Goiás (García-Melo et al. 2009) and high virulent strains that cause severe lesions in mouse brains including NC-Spain 5H, NC-Spain 7, and NC-Spain 9 (Pereira García-Melo et al. 2010). Consistent with previous work (Costa et al. 2018), the gerbils did not present any clinical signs or histological lesions during the time of infection.

3.5. Concluding remarks

In the present study, we demonstrate for the first time the isolation of two *N. caninum* goat strains, NC-goat1 and NC-goat2, and the pathogenicity in a BALB/c model of infection, with the NC-goat1 strain being considerably more virulent than the NC-goat2 strain. This study contributes to the behavioral understanding of two new *Neospora caninum* strains in the BALB/c mouse model; however, this study also unveils the question of whether or not the mouse model accurately represents the disease in naturally infected goats. Therefore, studying goats as an intermediate host of the parasite remains important because the strains present mid and low virulence in BALB/c mice but are already correlated with abortion in naturally infected goats. More studies are necessary to characterize the pathogenicity and virulence of the *N. caninum* strains from goats and the response of the caprine species to the parasite.

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Funding sources

We thanks to the Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) for a PhD grant (process number 2016/05010-2) and financial laboratorial support (process number 2016/24856-0) We would also like to acknowledge Fundação de Apoio à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the support on the field research of this work (process number CVZ-PPM 00763-16).

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

3.7 Tables

Table 1: The distribution of histological lesions in the brain of mice infected with the Nc-goat1 and Nc-goat2 strains.

strain	week	frontal cortex	basal nuclei	diencephalo	hippocampus	parietal cortex	mesencephalon	rostral colliculus	occipital cortex	pons	cerebellum	medulla
NC-goat1	1	-	-	-	-	-	-	-	-	-	-	-
	2	-	2/6 +	2/6 +	-	-	2/6+	-	-	-	4/6 +++	2/6 +++
	3	4/6 ++	3/6+	2/6+	2/6+	4/6++	2/6+++	4/6+	2/6+	5/6++	4/6++	4/6+++
	4	3/6++	2/6+++	4/6+	-	4/6+++	4/6+++	-	2/6+	-	6/6++	2/6+
	5	3/6+++	2/6+++	2/6++	1/6+	6/6++	-	4/6++	-	2/6+	6/6+	3/6+++
NC-goat2	1	-	-	-	-	-	-	-	-	-	-	-
	2	4/6+	-	2/6+	-	-	-	-	-	-	2/6+	-
	3	-	1/6+	2/6+	1/6+	1/6+	-	-	-	-	-	-
	4	1/6+	-	1/6+	1/6+	3/6+	2/6+	-	-	-	-	-
	5	-	4/6+	2/6+	-	2/6++	-	-	-	4/6+	1/6+	-

The lesions were composed of encephalitis, meningitis and perivascular cuffs and categorized as discrete ($^{+}$), moderate ($^{++}$) or severe ($^{+++}$).

Table 2: Immune-histochemical phenotyping of the cells in the CNS inflammatory areas.

		marker				
strain	week	IBA1	CD3	CD79-α	MHC-II	GFAP
NC-goat1	1	-	-	-	-	-
	2	+++	+++	+	+	-
	3	+++	+++	+	++	+
	4	+++	+++	+	++	+
	5	+++	+++	+	+++	+
NC-goat2	1	-	-	-	-	-
	2	+	+	-	-	-
	3	+ + +	+ +	-	+	+
	4	+ +	+ +	-	+	+
	5	+ +	++	-	+	-

The lesion intensity was classified by the average number of marked cells and is reported as low ($^{+}$; less than 10 cells), medium ($^{++}$; more than 10 cells, but as single focuses or less than 1 layer of perivascular cuff cells), or high ($^{+++}$; more than 10 cells in multifocal areas in the same brain region).

Microsatellite genotyping of *Neospora caninum* isolates from naturally infected goats

4 Microsatellite genotyping of *Neospora caninum* isolates from naturally infected goats

This chapter presents results obtained in an international internship and research program, held at the Moredun Research Institute in Scotland.

Abstract

Neospora caninum is considered to be the major pathogen that causes abortions in cattle worldwide and it can cause abortions also in sheep and goats. Since the description of the parasite in 1988, many studies have shown clinical and experimental results, both *in-vivo* and *in vitro*, that demonstrate different biological behaviour of *Neospora* isolates, suggesting that different strains, causing neosporosis, may have different degrees of virulence. The genotypes of several *N. caninum* isolates and strains from clinical samples have been analysed and the increasing number of different genotypes can further contribute to the identification of markers that could predict the clinical outcome of neosporosis in domestic animals. Studies from our group have shown that the outcome of neosporosis during gestation in goats can be variable, with different intensity of lesions and evidence of infected goats that don't transmit the parasite to their offspring were obtained. Samples from aborted bovine foetuses from the same region in Brazil and also from Dumfries & Galloway in Scotland were included in this study. Multi locus genotyping, presented here, utilized two distinct sets of microsatellite markers. Neighbour-joining and goeBURST analysis of the multi locus genotypes revealed that the isolates and clinical samples from goats are close related to each other but are not closely related to the genotypes found in bovine samples from the same region. The majority of the Scottish *Neospora* genotypes grouped together, which is in accordance with findings of other studies using Scottish samples. The present data show for the first time the results of multi locus genotyping using microsatellite makers for *N. caninum* samples from a population of goats in Brazil and provide evidence of mixed *N. caninum* infections in this goat population, contributing to the understanding of *Neospora caninum* diversity in this host species.

Keywords: Apicomplexa, *Capra hircus*, abortion, *Neospora*, microsatellite markers, mixed infections.

4.1. Introduction

The apicomplexan parasite *Neospora caninum* is considered the major agent that causes abortions in ruminants worldwide (Dubey and Schares, 2011), causing significant economic losses that were estimated at up to U$1,298 billion annually (Reichel et al., 2013). Besides cattle, other animals can act as intermediate hosts, like goats, sheep, buffalos, small mammals, birds and even the dog (Dubey and Schares, 2011). The parasite was first described in 1988 as an agent similar to *Toxoplasma gondii,* causing neuromuscular disease in dogs. Since then the parasite was demonstrated in several species and experiments using different isolates shows that those can have different behaviours in models of experimental infections both in vivo and in vitro. Those results suggest that different isolates can have different genetic factors that influence on their pathogenicity and virulence (Dubey et al., 2017). Viable *N. caninum* has been isolated from cattle, dogs, sheep, goats, water buffalo, horses, white tailed deer and axis deer. These isolates have variable levels of pathogenicity and virulence. Only a few of these isolates have been studied in more details, and these studies can largely explain the understanding of neosporosis regarding parasite virulence, immunogenicity, pathogenicity and other variables that are different between strains (Dubey et al., 2017).

The *N. caninum* genome (Nc-Liv isolate) was first described in 2012, demonstrating that *N. caninum* is different from *Toxoplasma gondii*, but with a high degree of orthology with very few chromosomal rearrangements between them. Beside whole genome sequencing, the use of microsatellite markers is another alternative to characterize the genotypes of new strains. These microsatellite markers are repetitive DNA sequences in the genome of eukaryotic organisms that are highly pleomorphic in sequence and length and may indicate differences in pathogenicity of distinct *N. caninum* strains. Regidor-Cerrillo et al. (2013) described 12 Msats (Sp-MS markers) and analyzed the genotypes of several *N. caninum* isolates and strains from clinical samples, showing that different clusters of multi locus genotypes of *N. caninum* are related to geographic origins of these isolates (Regidor-Cerrillo et al., 2013; Regidor-Cerrillo et al., 2006). Moreover, microsatellites are also a powerful tool to prove that new isolates are not derived from laboratory contaminations (Dubey et al., 2017).

The discovery of additional markers can contribute to the reliability of the clustering techniques, especially if the markers are located on chromosomes not yet covered by Msat markers, or on the apicoplast DNA (Regidor-Cerrillo et al., 2013). Guido (2013), described 12 novel Msat markers, identified at the Moredun Research Institute in Scotland (from now on referred to as MRI-MS markers). The study showed potential of samples clustering when clinical samples from bovine abortions in Scotland were analyzed, but more studies are necessary to evaluate the contribution of the MRI markers when analyzing samples together with the previously identified MS markers.

Microsatellite analysis reveal a broad range of different genotypes of *N. caninum,* but to the date there is no evidence of any association of allele patterns to strain virulence (Campero et al., 2018; Regidor-Cerrillo et al., 2006). Most of the genotyped strains were isolated from cattle. Recently, a goat strain has been genotyped, and the authors considered the possibility that this isolate is a goat adapted *N. caninum* strain, but it would require more isolates to be studies (Campero et al., 2018).

Clinical neosporosis manifests in the intermediate hosts as recurrent abortions, low pregnancy rate, neurologic signs and low average birth weight and clinically health but persistently infected animals (Dubey et al., 2017). As in cattle, there are reports of neosporosis in goats. *Neospora caninum* infection in goats can lead to abortions, stillbirths, clinically healthy but infected kids and the birth of uninfected animals despite their mothers being infected (Costa et al., 2014; Mesquita et al., 2013).

A few reports show the outcome of neosporosis in naturally infected goats, these consist mainly of sporadic cases (Campero et al., 2018; Costa et al., 2014; Mesquita et al., 2013). Goats were also used as experimental models of infection and they are showing similar placental lesions as infected cattle (Porto et al., 2016).

4.2. Background

Studies from our group show that the outcome of neosporosis during gestation in goats can be variable, with different intensity of lesions, and even reports of infected goats that don't transmit the parasite to their offspring (Mesquita et al., 2013). Placental and brain lesions can also be different depending on the goat family involved (Costa et al., 2018; Costa et al., 2014; Mesquita et al., 2013), furthermore, brain lesions, found in adult male goats, were more severe and more frequent compared to those in female

goats (Costa et al., 2014; Nakagaki et al., 2016). Recently we made two distinct *N. caninum* isolates from an experimental goat flock at the University of Lavras. The goats from this experimental flock came from different properties in southern Minas Gerais-Brazil and as a result multiple strains could be present The two isolates presented different levels virulence in a BALB/C mouse model of infection (Costa et al., 2019a). Identification of the genotypes of the 2 isolates with different degrees of virulence can help to deterimine how distinct genotypes can influence the outcome of infection during gestation.

4.3. Objectives

- Amplify microsatellite sequences from caprine and bovine samples from Brazil and Scotland using markers described by the group in Spain (Sp-MS markers) and the group in Scotland (MRI-MS markers).

- Characterize the microsatellite multi locus genotypes (MLG) of the two *N. caninum* isolates from goats and from placental samples from the goats in our experimental flock, which can transmit the parasite to their offspring for at least five generations.

- Characterize the *N. caninum* Msat pattern of aborted bovine fetuses from Minas Gerais in Brazil and from Dumfries and Galloway in Scotland.

- Compare all Msat patterns with previously described ones, and analyze them phylogenetically, to determine the relationship between sample hosts, geographical distribution and possibly virulence levels.

4.4 Materials and methods

4.4.1 DNA samples

In this study a total of 127 samples were analyzed, comprising 2 *N. caninum* isolates obtained from goats (Costa et al., 2019), 32 placental samples from naturally infected goats, 29 samples from aborted bovine fetuses from Minas Gerais in Brazil, and 64 samples from aborted bovine fetuses from Dumfries and Galloway in Scotland. All Samples were previously found to be positive for *N. caninum* by molecular and pathological analyses.

Clinical samples from goats

The 32 placental samples analyzed, came from 23 naturally infected goats. These goats were obtained for previous studies that describe caprine neosporosis

(Costa et al., 2018; Costa et al., 2019b; Costa et al., 2014; Mesquita et al., 2013; Nakagaki et al., 2016). All goats were maintained, in an experimental herd at the University of Lavras, Minas Gerais, Brazil and were monitored for at least 3 generations of kids. Female goats from different farms in southern Minas Gerais were introduced into the experimental flock during the years of 2009 and 2010. Female goats serologically negative to *N. caninum* were also kept in the same paddock, routine IFAT tests for *N. caninum* antibodies were taken from the whole flock for the duration of the previous experiments.

N. caninum isolates from goats

Viable *N. caninum* isolates were obtained from two different goats from the same experimental herd (Costa et al., 2019). The first from a placental sample of a goat with previous history of abortion, and the second from the brain of a congenitally infected male goat. For the parasite isolation from both animals the process included an initial bioassay in gerbils, followed by a second bioassay in IFN-γ KO C57BL6 mice and subsequent culture of tachyzoites in monolayers of VERO cells (Costa et al., 2019).

Clinical samples from aborted bovine foetuses

Bovine abortion samples from a previous study in southern Minas Gerais, Brazil were analysed (Orlando et al., 2013). Samples were obtained during 2011 to 2013; they were sent by local farmers to the Veterinary Pathology laboratory of the Federal University of Lavras (MG-Brazil). *Neospora caninum* diagnosis was previously confirmed by PCR, histopathological, and immunohistochemical diagnosis (Orlando et al., 2013).

Samples from aborted bovine foetuses from Scotland were also included in this study. A total of 64 samples, previously confirmed for the presence of *N. caninum* ITS1 DNA, were analysed (Bartley et al., 2009). All samples originated from 13 farms in the Dumfries and Galloway region in south-west Scotland and were collected between March 2008 and June 2011.

Reference Isolates

DNA samples from isolates with previously described Msats patterns were used as a reference, the isolate NC-SP1 (Oliveira et al., 2017) was kindly provided by

Solange Oliveira (University of São Paulo- SP, Brazil.), and two Laboratory maintained cultures of the Strain NC-1, kindly provided by Luis Miguel Ortega-Mora (SALUVET, University Complutense of Madrid- Spain) and Gereon Schares (Friedrich-Loeffler-Institut, Insel Riems, Germany).

Samples from the isolate "NC1/Liv-c" cultured at the Moredun Institute in Scotland since 1993 was also analyzed at different passages: 10, 20, 38, 84, 100, 134, 178, 217, 287, 335.

4.4.2- Neospora caninum ITS1 PCR

Total DNA was extracted from tissues of goats, aborted calves or cell cultures using a commercial kit (Reliaprep gDNA Tissue Miniprep System – Promega). For the confirmation of *N. caninum* DNA in the samples, the *N. caninum* ITS1 nested PCR was performed as previously described by (Bartley et al., 2009). PCR reactions were run in triplicates and the products of the second reactions analyzed by agarose gel electrophoresis.

4.4.3- Amplification of the Sp-MS markers

From the 12 Microsatellites markers described by Pedraza-Díaz et al., (2009), 9 with a higher discriminatory power were used: MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21. Nested PCR reactions were carried as previously described (Regidor-Cerrillo et al., 2006) with minor modifications. The first PCR rounds were carried out in two multiplex reactions, the first using the primers for the Msats MS4, MS5, MS7, and MS21, and the second with the primers for Msats MS6a, MS8, MS10, and MS12. The first rounds of PCR, for the two sets of multiplex primers, consisted of 2.5 μL 10x PCR buffer, 0.2μM of each forward and reverse primer for each marker, 2.5 units of Taq Polymerase and up to 300ng of template DNA, in a final volume of 25μL. PCRs were performed with an initial cycle of denaturation at 95°C for 1 minute, 35 cycles of denaturation (95°C for 1 min), annealing (53°C for 1 min) and elongation (72°C for 1 min), followed by a final extension cycle at 72°C for 10 min. PCR products from the first reaction were diluted with 100μL of distilled water and 5μL of this were used as template for the second reaction. The second round PCR reactions followed the same conditions as the first round with minor modifications: each reaction used just a single pair of primer pair for each Msat marker with the forward primer labeled with an appropriate fluorophore as described in section 5.5. The final volume was adjusted

to 20µL. PCRs for the Msat marker 6b were performed without the presence of other primers.

4.4.4- Amplification of MRI-MS markers

Guido (2017), described 12 novel Msat markers for *N. caninum*, 6 of them having a higher discrimination power for the analyzed samples (MRI2, MRI7, MRI14, MRI37, MRI41 and MRI42). Nested PCR reactions were performed as described in section 5.3 with minor modifications. The first round PCRs were performed as a multiplex reaction using 0.2ng of each forward and reverse primers for each of the 6 MRI-MS markers. The annealing temperature for the first PCR round was 57°C for 1 min. PCR products from the first reaction were diluted with 100µL of distilled water and 5µL used as template for the second reaction. The second round PCRs had the same conditions as the first round with minor modifications: each reaction used just a single set of primers for each Msat marker, with the forward primer labeled with an appropriate fluorophore as described in section 5.5. Final volumes were adjusted to 20µL. Annealing temperatures were 57°C for markers MRI37, MRI41 and MRI42, and 58°C for markers MRI2, MRI7 and MRI14.

4.4.5- Capillary electrophoresis (CE)

Labelled PCR products were subjected to fragment analysis using capillary electrophoresis via an ABI 3730 DNA Analyzer (Applied Biosystems), using size standard Genescan ROX500 (Applied Biosystems). The second round of each Msat PCR product was analysed in an agarose gel electrophoresis. Positive DNA bands were classified as strong or weak. All strong positive reactions were diluted 1:100 in distilled water and weak reactions diluted 1:50. All compatible samples were multiplexed in a well, using 1µL of each diluted PCR product labelled with FAM, 2µL of products labelled with HEX and 3µL of each product labelled with NED, the final volume was adjusted to 10µL. Samples that were negative at the gel electrophoresis were submitted as a single sample, in a final dilution of 1:10.

For multiplex analysis, Msats of different sizes were analysed together, Msats that overlap in size were submitted to CE with different fluorophores. All 15 Msats for each sample were sent to CE using initially 3 wells, the Msats were mixed as follows:

1- MRI41(HEX); MS6b(HEX); MS4(FAM); MS12(HEX); MRI7(NED); MRI37(FAM)

2- MRI42(FAM); MS5(FAM); MS6a(HEX); MS10(NED); MRI2(FAM)

3- MS8(FAM); MS21(FAM); MRI7(NED); MRI14(FAM)

The Msats MS12 and MS6b were analysed as single reactions for some of the *Neospora* samples wherever they overlapped. Electropherogram results were analysed in the software Geneious Prime® (www.geneious.com), using a microsatellite plugin. Allele numbers for each of the Msats were assigned to the samples according to the sizes determined by Regidor-Cerillo. Possible changes in fragment size determination regarding reaction conditions, different analysis instruments or other laboratory-related variables were adjusted following the results from the reference isolates.

4.4.6- PCR product cloning and sequencing

To confirm the CE size determination and allele designation, PCR products of each Msat were sequenced and used as reference. Possible new alleles, according to CE, were also sent for sequencing and when a complete fragment sequence was not obtained, amplicons were cloned and sent for sequencing.

The cloning procedure followed a fragment insertion protocol for the pGEM®-T Easy Vector (Promega). After ligation of PCR products into vectors, JM109 competent *E. coli* cells were transformed with the vector-insert construct and screened using LB/ampicillin/IPTG/X-gal plates. This generates white colonies, representing a successful DNA insertion, and blue colonies without inserts. Successful cloning disrupts the formation of functional □-galactosidase resulting in white colonies and if functional □-galactosidase is produced then the colonies are blue. After incubation at 37°C overnight, white colonies where selected, plated again on LB/ampicillin/IPTG/X-gal plates, and incubated overnight. A small sample of colonies for each Msat marker was harvested, put into 50µL of distilled water, incubated at 95°C for 15 minutes and used as a template for the according Msat PCR amplification to confirm the presence of the fragment of interest. For DNA sequencing, selected colonies were grown overnight in 10ml agar L-Broth at 37°C with agitation. On the following day cells were pelleted by centrifugation at 10000G for 10 min and plasmid DNA extracted using the kit Wizard® Plus SV Minipreps DNA Purification System (Promega). 300ng of DNA was used for sequencing reactions with the T7 and SP6 primers, which amplify the whole fragment inserted into the vector.

4.4.7- Clustering analysis

To determine proximity between the samples analysed, a Neighbour-Joining dendrogram was built using the 9 MS MSat markers from the samples studied in combination with other worldwide samples with MSat patterns described by (Regidor-Cerrillo et al., 2013). For this purpose, the software Phyloviz-2.0a was used (Francisco et al., 2012; Nascimento et al., 2017), population diversity was estimated using Hamming Distance in Saitou-Nei criterion. Only samples with at least 6 determined MS Msats alleles were included in the analysis. A Global Optimal eBURST analysis was also made to determine proximity between samples analyzed (Francisco et al., 2009). Complementary analysis was also done using the same samples in a Global Optimal eBURST. Neighbour joining analysis considered samples with missing alleles like previously reported by Regidor-Cerrillo et al. (2013). The goeBURST analysis excluded samples with missing alleles and also substituted those missing alleles with arbitrary alleles for the missing data points.

4.5. Results

CE peaks analysis: All 15 microsatellite markers could be visualized in 3 multiplex readings (**Figure 1**). For some samples and the NC-1 isolates, the amplicon sizes of Sp-MS MS6a and MS12 overlapped with each other or with other amplicons and they were reanalysed as individual amplicons. *Sp-MS analysis of clinical samples and isolates from goats*: Out of the 32 goat samples analyzed, a complete MLG could be obtained for only 7 samples. All samples with incomplete MLGs were excluded due to lack of variation among the alleles obtained. All the samples included in the final analysis are listed in **Table 1**. The **Table 2** presents the alleles assigned to each locus of the respective sample. The goat samples G1 and G2 were obtained from mother and daughter respectively and they had the same MLG. However, they had a different MLG compared to that from isolate NC-Goat1 (obtained from the same placental tissue from sample G1), they differed by 3 Sp-MS alleles. The MLGs from samples G1 and G2 were identical to that of isolate NC-Goat2 when comparing the Sp-MS MLGs. The Nc-Goat2 isolate was obtained from a brain sample of a male goat, which is the son of the goat from which the placental sample G4 was obtained. Goat sample G4 was a daughter of goat G3 and they both have the same MLG, but it was slightly different from the MLG of the NC-Goat2 isolate, they differ by 2 Sp-MS alleles. In Summary, 6 different MLGs were identified within the goat clinical samples and isolates.

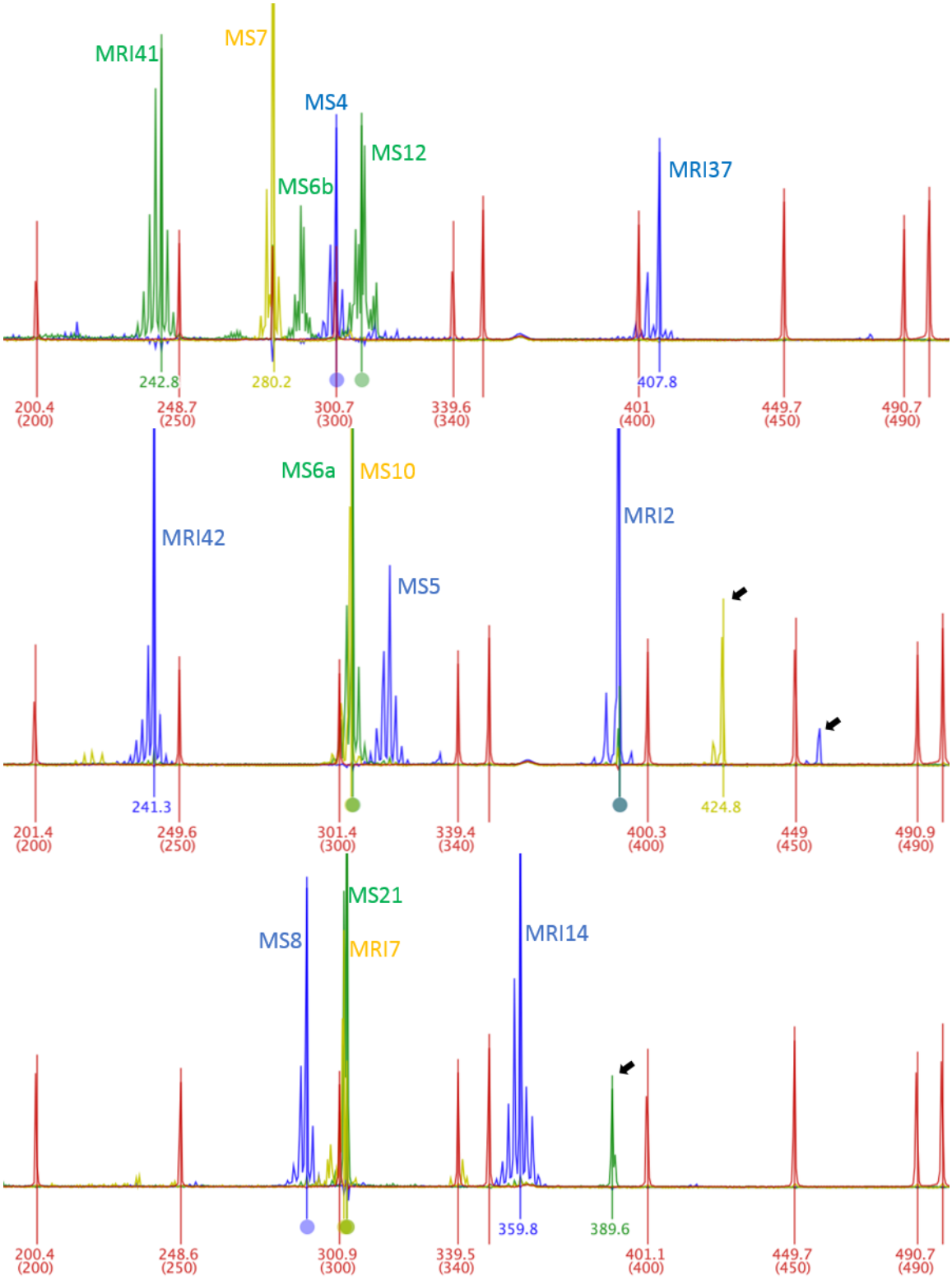

Figure 1: Multiplex electropherograms for Microsatellite markers of the isolate NC-Goat2. All 15 Msats are clearly visible when analysed as three different mixes, each marker is labelled with an appropriated fluorophore, represented in different colours: FAM (blue), HEX (green), NED (yellow) and the size standards in ROX (red). Occasional unspecific peaks can appear (arrows) but didn't interfere with the analysis.

Table 1: Samples included in this study with information on origin (country and region), animal host species and breed from which they were obtained. NK denotes not known.

Sample ID	Country	Region	Host Sampled	Breed
NC-Goat1	Brazil	Lavras-MG	goat	NK
NC-Goat2	Brazil	Lavras-MG	goat	NK
G1	Brazil	Lavras-MG	goat	Mixed
G2	Brazil	Lavras-MG	goat	Mixed
G3	Brazil	Lavras-MG	goat	Saanen
G4	Brazil	Lavras-MG	goat	Saanen
G5	Brazil	Lavras-MG	goat	Saanen
G6	Brazil	Lavras-MG	goat	Alpine
G7	Brazil	Lavras-MG	goat	Alpine
B1	Brazil	Lavras-MG	cattle	Holstein
B2	Brazil	Lavras-MG	cattle	Holstein
B3	Brazil	Lavras-MG	cattle	Holstein
B4	Scotland	Dumfries/Galloway	cattle	NK
B5	Scotland	Dumfries/Galloway	cattle	NK
B6	Scotland	Dumfries/Galloway	cattle	NK
B7	Scotland	Dumfries/Galloway	cattle	NK
B8	Scotland	Dumfries/Galloway	cattle	NK
B9	Scotland	Dumfries/Galloway	cattle	NK
B10	Scotland	Dumfries/Galloway	cattle	NK
B11	Scotland	Dumfries/Galloway	cattle	NK
B12	Scotland	Dumfries/Galloway	cattle	NK
B13	Scotland	Dumfries/Galloway	cattle	NK

Table 2: Microsatellite MLG results for *N. caninum* samples from goats and cattle. Assignment of alleles for each correspondent locus or size determination of capillary electrophoresis when it was not possible.

Strain	MS4	MS5	MS6a	MS6b	MS7	MS8	MS10	MS12	MS21	MRI2	MRI7	MRI14	MRI41	MRI42	MRI37
Nc-Goat1	13	12	15	11	9.1	12	6.14.10	16	6	7	310	362	240	231	7
Nc-Goat2	13	16	15	12	9.1	13	6.14.10	16	6	3	302	359	243	240	7
G1	13	16	15	12	9.1	13	6.14.10	16	6	3	301	360	240	239	7
G2	13	16	15	12	9.1	13	6.14.10	16	6	3	301	360	240	239	7
G3	13	18	17	12	9.1	13	6.14.10	16	6	4	302	362	243	241	8
G4	13	18	17	12	9.1	13	6.14.10	16	6	4	301	362	239	238	8
G5	12	16	15	12	9.1	13	6.14.10	16	6	3	302	360	303	239	7
G6	13	19	17	12	9.1	13	6.14.10	16	6	4	301	362	239	239	7
G7	13	15	15	13	9.1	13	6.15.8	16	6	5	301	NA	238	239	NA
B1	13	17	16	13	NA	13	NA	16	6	NA	NA	370	242	236	4
B2	13	19	NA	304	NA	13	NA	17	6	NA	NA	NA	NA	NA	NA
B3	14	10.13	15	NA	9.1	12	NA	17	6	6	309	361	NA	232	7
B4	12	7	13	13	11	14	307	14	6	NA	287	365	241	244	6
B5	12	9	13	11	17	11	307	14	6	5	299	367	247	241	NA
B6	11	10	13	12	9.1	19	310	15	6	6	320	363	243	235	NA
B7	NA	8	13	12	13	11	307	14	6	6	317	363	245	235	NA
B8	11	10	13	NA	9.1	17	311	17	6	6	295	375	247	243	NA
B9	11	NA	13	11	9.1	17	311	17	6	6	299	375	249	241	NA
B10	11	10	13	11	9.1	NA	311	16	7	5	291	373	247	237	NA
B11	11	10	13	11	9.1	18	NA	NA	6	5	293	370	247	237	NA
B12	11	10	15	12	9.1	18	NA	NA	6	NA	291	NA	243/247	237	NA
B13	13	10	14	12	15	15	NA	NA	6	NA	291	NA	248	233/239	NA
NC-SaoPaulo1	13	19	15	12	9.1	13	6.14.10	16	6	3	NA	358	240	293	NA
NC-1(Spain)	12	12	12	17	16	16	7.12.9	16	303	2	313	383	247	247	9
NC-1(Germany)	12	12	12	17	16	16	7.12.10	17	303	2	318	391	235	247	9

Allele assignment for MS markers according results published by Regidor-Cerrillo et al. (2013). NA= allele not assigned. MRI markers 7, 14, 41 and 42 presented as the fragment size determined by capillary electrophoresis.

Clinical samples from aborted bovine foetuses from Brazil: From the 29 aborted bovine foetal samples analysed a partial Sp-MS MLG was obtained for two samples. For these either 2 or 3 alleles for the 9 loci could not be determined. Interestingly there is evidence of a mixed infection for bovine sample 3 from Brazil, which had 2 peaks for Sp-MS MS5.

Clinical samples from aborted bovine foetuses from Dumfries and Galloway in Scotland: A complete Sp-MS MLG was obtained from 3 of the Scottish samples (B4, B5 & B6), and a partial Sp-MS MLG from 7 other samples (B7-13). These were missing either 1 or 2 alleles for the 9 Sp-MS loci. All Scottish samples in this study presented a weak band or no band for MS10 in the agarose gel electrophoresis analysis. Sequencing results of MS10 amplicons was not achieved and their results are presented as their peak sizes determined by capillary electrophoresis in **Table 2**.

Sp-MS performance:

Clustering analysis: In the Neighbour joining dendrogram (**Figure 2**), the *Neospora* MLGs from goats are separated into two different groups: the first one is represented by the isolate NC-Goat2 and includes clinical samples G1-G6; and a second group represented by the isolate NC-Goat1, which includes the clinical sample G7. For the bovine foetuses from Brazil the MLGs were different from the MLGs of the goat samples, and in the neighbour joining analysis they are separated from the goats and were located on a different branch of the tree. When analysing the genotypes from the aborted foetuses from Scotland 9 of the MLGs grouped together in the phylogenetic tree, in close proximity with other Scottish samples from the same region previously described by Regidor-Cerillo et al. (2013). Only the sample B13was located on another branch of the tree, but in a relatively close proximity to two other Scottish samples that were previously described.

The goeBURST analysis (**Figure 3**) reveals a different relationship of the *N. caninum* MLGs from goats. All, but one, are closely related and group with another 2 isolates from Brazil (NC-SaoPaulo1 and NC-Goias). Interestingly the MLG of sample G7 is relatively closely related to an Argentinian MLG that was found in a goat. The bovine samples from Brazil, are not very closely related to the goat samples and grouped differently than with the Neighbour joining analysis. Sample B3 is located in a different position and is not closely related to the other bovine samples from Brazil. In

accordance with the Neighbour joining analysis, the majority of the Scottish samples are grouped together with a few exceptions and these are closely related with other Scottish MLG which were previously described.

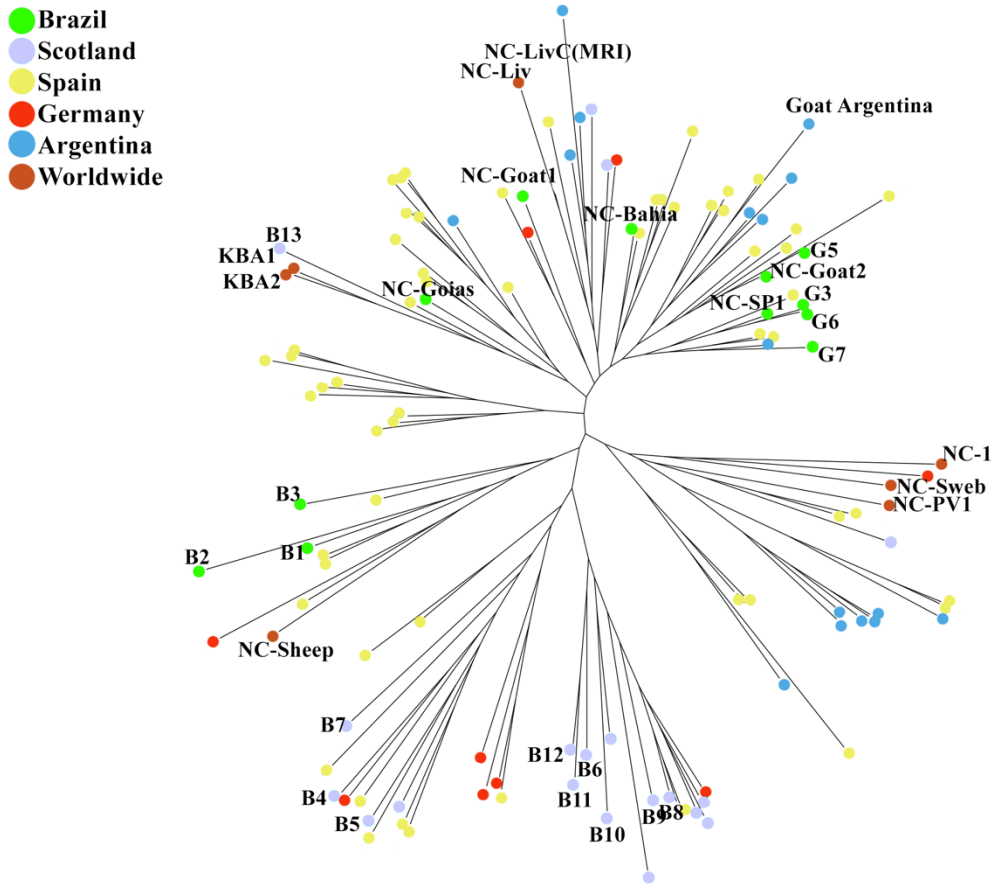

Figure 2: Clustering of *Neospora caninum* MLGs based on Sp-MS markers. Neighbor joining dendrogram showing the relationship between *N. caninum* samples from around the world. Each country of origin of the samples is represented by a different color and reference samples from across the world are highlighted as Worldwide.

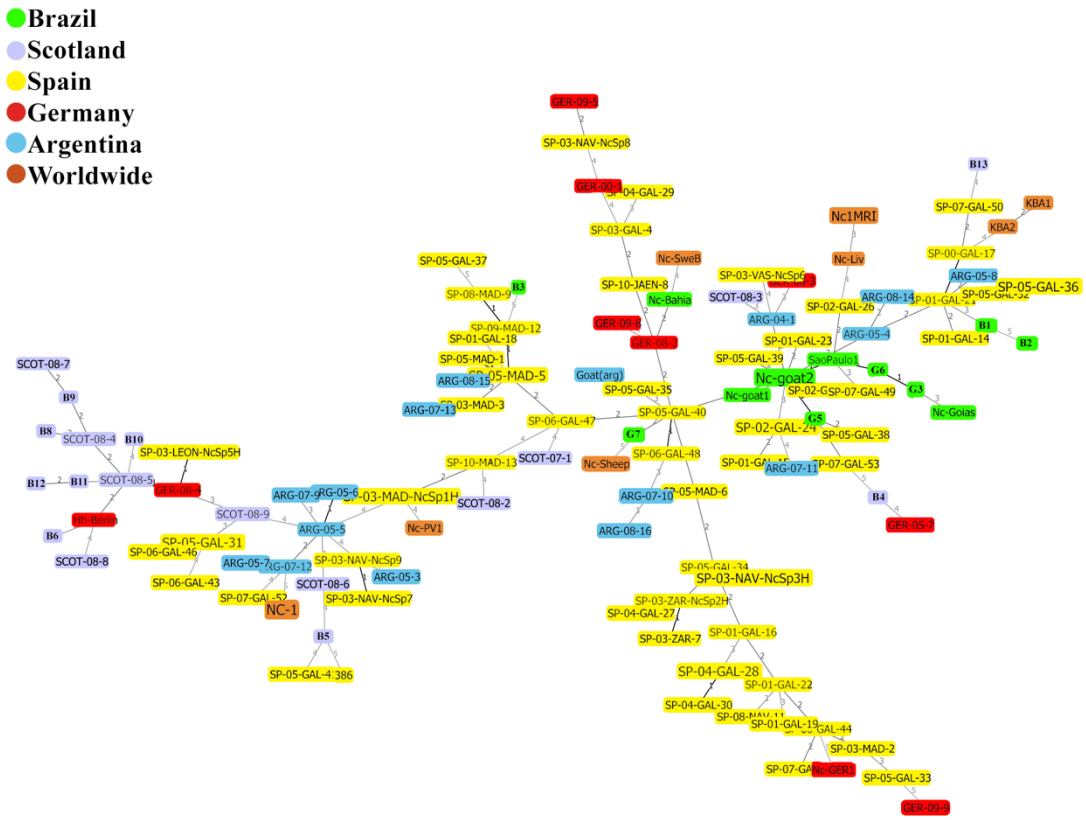

Figure 3: goeBURST graph showing the relationship between *Neospora caninum* genotypes from around the world. Each country of origin of the samples is represented by a different colour and reference samples from across the world are highlighted as Worldwide.

MRI-MS Genotyping: The microsatellites MRI41 and MRI42 were variable between samples G3 and G4, previously described as being identical according to the Sp-MS MLGs. However, the marker MRI41 also presented different fragment sizes for the NC-1 isolates from Germany and Spain, the same also occurred for the markers MRI7 and MRI14 for these isolates. The MRI41 presented clear evidence of microsatellite instability for the NC-1 isolate from Spain, by Capillary electrophoresis analysis (**Figure 4**), showing a peak with a “tail” suggesting a shift to another allele within this sample.

The patterns of all peaks for every sample were evaluated to rule out instability or double peaks. All markers had stutter bands surrounding the main peak. All peaks for each Msat marker resulted in unique MLGs with minor changes between different

samples (**Figure 5**). Some markers presented secondary peaks for the isolates NC-1 (German and Spanish samples) and NC-SaoPaulo1. This phenomenon was more frequently observed with the MRI-MS markers but also present for the Sp-MS markers. Only the predominant peaks were considered for each sample/locus combination.

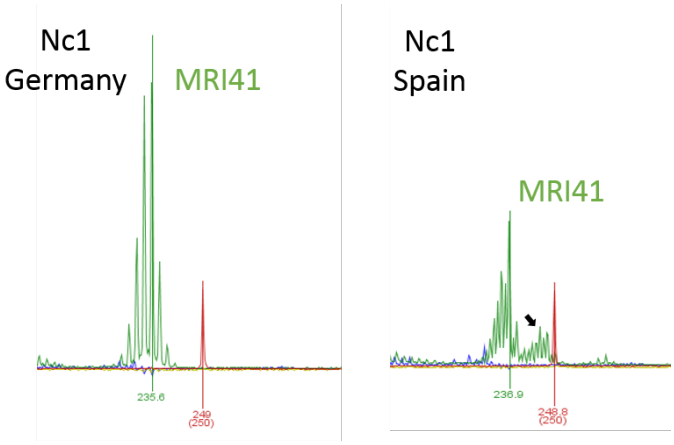

Figure 4: MRI41 electropherogram for the NC-1 isolate from Germany and Spain. The marker presents clear signs of microsatellite instability for the Spanish sample, characterized by a lower predominant peak followed by an additional secondary peak (arrow).

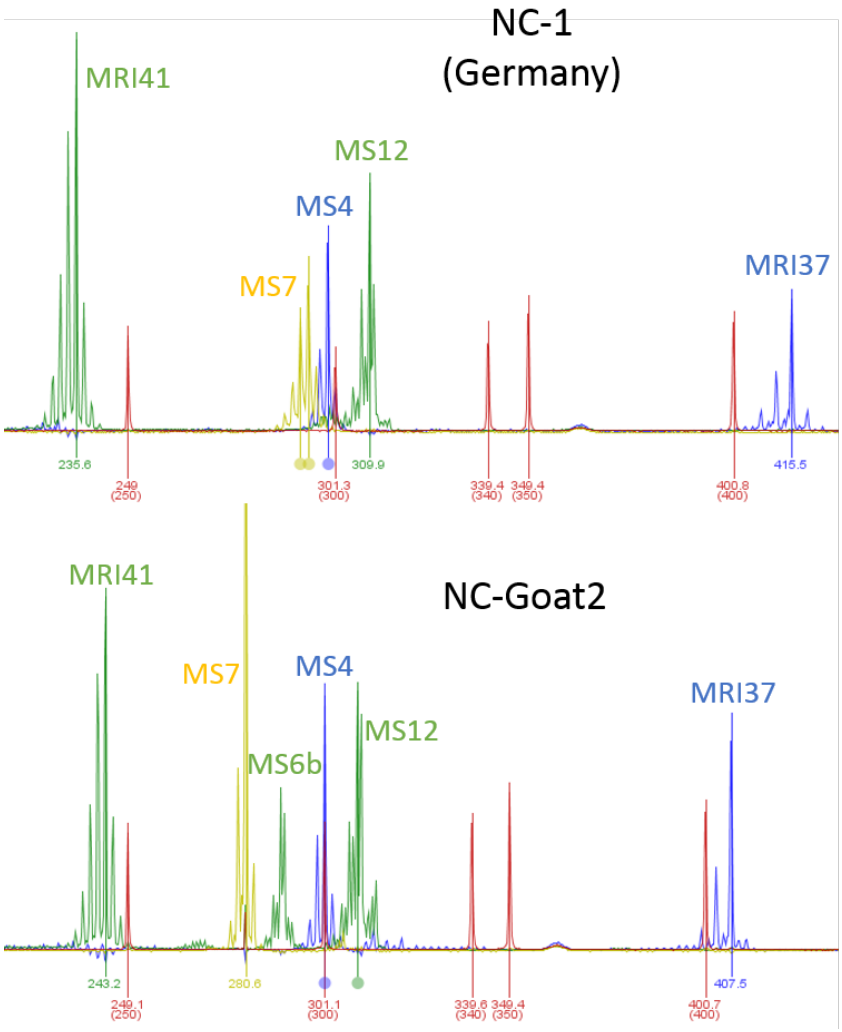

Figure 5: Electropherograms of the first Msat mix for isolates NC-1 (Germany), and NC-Goat2. Note that besides different sizes in markers from the two samples, they have a similar pattern for the respective marker. The mix for NC-1 (Germany) was analysed without the marker MS6b, which overlapped the marker MS12.

The Sequencing analysis for the MRI7 presented the repeat pattern $(TACA)_{13}TATA(TACA)_4TACG(TACA)_{14}$ for the NC-1 sample from Germany, and the pattern $(TACA)_{13}TATA(TACA)_4TACG(TACA)_{13}$ for the sample from Spain. The sequence repetition pattern obtained from these samples also differs from that described previously by (Guido, 2017), where it is presented as $(TACA)_XTACG(TACA)_Y(AT)_Z$.

The MRI42 Msat is described having the repetition sequence $(TAGA)_X(TA)_Y$, but when sequenced for the NC-LivC isolate, it presented the pattern

$(TAGA)_{16}(TAGATA)_7(TAGA)_3TA(TAGA)_3GA(TAGA)_3$. Also, MRI-MS fragment sizes were variable for different passages of the isolate Liv-c (Moredun) (**Table3**).

Table 3: Fragment size determination by Capillary electrophoresis for different passages of the isolate Liv-c(Moredun).

Passage	ms4	ms5	ms6a	ms6b	ms7	ms8	ms10	ms12	ms21	mri2	mri7	mri14	mri41	mri42	mri37
10	304	302	304	288		297		309	302	382		381		247	240
20	304	302	304	288		297		309	302	383/386		381		247	244
38	304	302	304	288		297		309	302	383		381		247	240
84	304	302	304	288		297		309	302	379/383		381		247	240
100	304	302	304	288		297		309	302	383/386		377/381		247	244
134	304	302	304	288		297		309	302	383		385		247	244
178	304	302	304	288		297		309	302	382/386		377		247	244
217	304	302	304	288		297		309	302	386		377		247	244
287	304	302	304	288		297		309	302	386		377		247	244/248
335	304	302	304	288		297		309	302	386		377		247	248

Sizing for the markers MS7, MS10, MRI7 and MRI41 were not performed.

4.7. Discussion

1- First microsatellite study in *N. caninum* samples from a population of goats from Brazil.

This study describes the use of microsatellite genotyping of *N. caninum* samples from a population of goats in Brazil for the first time. MSat amplifications of the 2 separate sets of markers (Sp-MS & MRI-MS) followed protocols described by Pedraza-Dias et al. (2009), and Guido (2018). An Msat MLG was obtained only for 21.8% of goat samples analysed, 10.3% of bovine abortion samples from Brazil and 15.6% of bovine abortion samples from Scotland, besides all samples being positive for *N. caninum* ITS1 DNA. It is expected that the MSat amplification have a lower sensitivity compared to amplification of the ITS1 region, as the ITS1 target is within the ribosomal DNA, which has multiple copies within each parasite. This results in a far superior sensitivity when compared to single copy gene targets (Dubey et al., 2017; Holmdahl and Mattsson, 1996). The poorer performance on the amplification of Msat markers is further confounded by low quantity of parasite DNA in the samples and poor sample conditions when collected, like in mummified or macerated foetuses (Regidor-Cerrillo et al., 2013). Nevertheless, the genotyping of some of the samples allowed us to make some inferences about the identity of *N. caninum* strains present in goats from Southern Minas Gerais in Brazil and how it compares to bovine samples from the same region and from around the world.

2- Neighbour joining and goeBURST analysis shows clustering of *N. caninum* genotypes according to its geographical origin

A neighbour joining analysis of the MLGs of the new samples, complemented with those of other samples genotypes from previous studies (Campero et al., 2018; Oliveira et al., 2017; Regidor-Cerrillo et al., 2013), showed that the goat genotypes are close related to each other and can be divided into two main groups. The first group it represented by the NC-Goat2 isolate and is closely related to the Isolate NC-SP1, and a second group led by the isolate NC-goat1 in a relatively close relationship with two other Brazilian isolates, NC-Bahia and NC-Goias. The three genotypes obtained from aborted bovine foetuses from Brazil are grouped on another branch of the tree, suggesting that these cattle could have been exposed to a different source of *N. caninum*. Most of the 11 analysed bovine samples from Scotland grouped together on the same tree branch with other Scottish samples, analysed by Regidor-Cerrillo et al. (2013). The fact that not all MSat alleles could be identified for the Scottish samples in the present study, did not invalidate the clustering analysis. Although considered unstable, the Neighbour joining analysis segregated samples according to geographical location (Pritchard et al., 2000; Regidor-Cerrillo et al., 2013). Campero et al., (2018) suggested that a conserved genotype could be involved in the infections of goats, but most of the *N. caninum* genotypes obtained from the Brazilian goats are not closely related to the *N. caninum* genotype found in one goat from Argentina. The goeBURST analysis presents similar findings to the Neighbour joining analysis. All but one Brazilian goat samples were in close proximity of each other and were closely related to the Brazilian isolates NC-SP1 and NC-Goias. One of the goat samples (G7) is in closely related to the goat genotype presented by Campero et al. (2018). Two of the bovine samples from Brazil are grouped together but they are not very closely related to the goat samples, while the 3rd bovine sample is more distantly related. Most of the Scottish samples are conserved in a group, with only a few exceptions. The Spanish and Argentinian genotypes seem to be more scattered across the graph, like previously presented by Regidor-Cerrillo et al. (2013). Both the Neighbour joining analysis and the goeBURST seems suitable to determine the relationship between *N. caninum* samples from all over the world. The results from both analyses are complementary and give a more complete picture when all samples were analysed, despite the missing allele designations for some samples. Missing allele information

could lead to artefacts in the analysis, like the non-grouping of all bovine samples from Brazil. But the fact that most of the Scottish samples and the goat samples were close related suggests that the use of samples with missing genotype information are not too detrimental for the goeBURST analysis in this study.

3- Microsatellite MLG profile analysis of goat samples reveals evidence of mixed infections with multiple strains.

The two goat isolates were generated from samples that originated from two distinct goat families. For each isolate there was a conserved MLG found in their respective mother and daughter samples but the MLG obtained for the isolates differed from the MLGs of the goat families the isolates were obtained from. In this study both isolates exhibited the same MS10 allele (6.14.10) but previous sequencing results of the MS10 alleles obtained during the generation of these isolates gave different results. These MS10 alleles also differed from those found in the goat families that the isolates were generated from. The MS10 amplicon for the NC-goat1 isolate was sequenced during the isolation process from infected gerbils (Costa et al., 2018) and the allele designation was 6.15.8, which is different to the allele (6.14.10) found in Nc-goat1 and in G1, which represents the source of the Nc-goat1 isolate. For NC-goat2 a MS10 amplicon was obtained and sequenced during the IFN-γ KO mouse bioassay and allele 6.13.12 was identified (unpublished data). However, the maternal sample and the Nc-goat2 isolate have MS10 allele 6.14.10. Obtaining different allele designations for samples obtained from a single goat at different stages during the isolate generation process provides clear evidence that the goats must have been infected with multiple *N. caninum* genotypes and that the isolates lost variability and became more clonal during successive passages in gerbils, KO mice, and cell cultures. Unfortunately, the exact samples from those previous analyses were no longer availed for the present study. Interestingly Nc-goat2 and samples G1 and G2 share the same MLG, however they represent the two separate goat families. Finding the same MLG within the two goat families suggest exposure to a common source of infection within the 2 distinct goat herds in the past or at least exposure to the same parasite genotype. Mixed infections with different *Neospora caninum* strains have been reported but is not well understood. (Prandini da Costa Reis et al., 2016), describes mixed infections with isolates that differed at the MS10 locus in two samples from a transplacentally infected dog pup. This was the first study to show a mixed *N. caninum* infection. The presence

of mixed genotypes in a single animal could result in the generation of new strains if infected meat, containing different isolates, is eaten by a naïve dog. The sexual cycle in the dog could then result in sexual crosses between different isolates and the generation of recombinants containing of genetic material from the parental genomes involved in the coinfection. These recombinants could have with distinctive genotypes and phenotypes as documented for the closely related protozoan *Toxoplasma gondii* (Grigg et al., 2001) thus changing the behaviour of the parasite during infections in an endemic region.

(Regidor-Cerrillo et al., 2013), describes a low percentage of samples that had multiple alleles in the CE analysis, but mostly it was considered as a transient mutation/shift, a phenomenon that is expected to occur when studying MSats (Schlötterer, 2000). In this study all isolates and clinical samples, presented stutter bands on the electropherograms. It is expected that additions and deletions of MSats repetitions can occur in a higher frequency when amplified *in vitro* by PCR (Hosseinzadeh-Colagar et al., 2016) and this phenomenon is seen at the electropherograms as secondary peaks that vary by the number of the repetition in the respective MSat (Ajzenberg et al., 2010; Regidor-Cerrillo et al., 2013). Usually the higher predominant peak is considered as the correct one and secondary peaks are disregarded unless they are at least 1/3 of the size of the predominant peak (Pacheco et al., 2019). As the life cycle stage of *N. caninum* analysed within those samples are haploid, just one peak at the electropherogram is expected (Regidor-Cerrillo et al., 2013). As the electropherograms obtained from other works on microsatellite genotyping of *N. caninum* are not shown (Pedraza-Díaz et al., 2009; Regidor-Cerrillo et al., 2013; Regidor-Cerrillo et al., 2006), we don't know for sure how often those secondary peaks can appear even when analysing theoretically clonal populations correspondent to the *Nesopora* isolates. Sometimes the analysis of peaks from the isolates NC-1 and NC-SP1 also revealed secondary peaks for some markers. The identification of mixed infections is reported for other parasites like *Malaria* (Pacheco et al., 2019) and *Theileria* (Katzer et al., 2010). In some of these works, the genotyping of single "isolates" was achieved by cloning the parasites, growing them after limiting dilutions of infected PBMC cells (Katzer et al., 2010). Sub-cloning of *N. caninum* isolates, especially from those with lower passage numbers, could reveal the presence of multiple genotypes during infections. Future analysis and optimizing of PCR

conditions should also be performed to investigate the origin of double peaks. Especially as previous studies on microsatellite genotyping of *N. caninum* don't specify the exact reagents used for the PCR conditions.

4- The MRI-MS markers are highly variable and could be used to differentiate samples from a same isolate or closely related isolates.

The MRI-MS markers identify more variability during the MLG analysis. Marker MRI37 is located on chromosome VI and marker MRI7 on chromosome XI, resulting in additional genome coverage for which no MS markers existed. The Msats MRI7, MRI41 and MRI42 exhibited with different peaks for samples of the same isolate (NC-1) that was obtained from different sources (Germany and Spain). MRI41 presented a sign of instability on the electropherogram, for the NC-1 sample from Spain.

When analysing the alleles for the MRI markers in different passages of the isolate Liv-c, a high frequency of changes in the size of the amplicon was observed. Comparison of the sequence composition of the MS markers revealed that the MRI markers have a considerably higher number of repetitions and are more complex. Previous studies have shown that the repeat number is positively correlated with an increase of the mutation rate in tandem repeats (Brinkmann et al., 1998; Schlötterer, 2000; Weber and Wong, 1993). Mutation rates in microsatellite sequences are considered to be several orders of magnitude greater than that in other DNA sequences (Schlötterer, 2000). The reasons why an MSat sequence could be more likely to mutate is uncertain. Weber and Wong (1993) showed that tetranucleotide repetitions are more mutable, otherwise (Chakraborty et al., 1997) shows that dinucleotides are more prone to mutate. Studying the behaviour of these markers in other *N. caninum* samples could contribute to the understanding of factors that lead to changes in MSat sequences in this parasite, and with the discovery of new genotypes those markers could be suitable to differentiate very close related isolates, or possibly used to predict changes in the parasite behaviour related to changes in its virulence as reported for long term passages (Bartley et al., 2006). The occurrence of MSat instability is also observed when analysing MSats from cancerous tissue samples in humans, where a high percentage of DNA replication defects can occur. Replication defects can be also influenced by other external factors such as exposure to pollution or radiation (Ellegren, 2000). All the 9 MS MSats in a study by Regidor-Cerrillo et al. (2013) showed to be stable when analysing 9 different samples of the NC-1 isolate

from different laboratories around the world, the alleles stayed consistent for all of them, except for the marker MS6b from one sample cultured in Balcarce (Argentina). More studies with a larger number of samples are necessary to confirm the suitability of MRI msats to the MSat genotyping method. Guido (2018) only analysed samples using the MRI markers but the combination of both MS and MRI markers is necessary to evaluate the suitability of both markers sets together to discriminate samples.

4.8. Conclusions

The present work shows genetic variability on *N. caninum* samples from goats and cattle from Brazil and cattle from Scotland. The results suggest that those goats were infected with mixed genotypes of the parasite. This is the first time that mixed infections by *Neospora canium* are shown in a population of animals. Sharing of identical genotypes by animals originating from different farms suggests that those goats were exposed to a common source of infection in the past or at least to a parasite with the same MLG. More studies are necessary to understand the implications of mixed infections in animals and how it could influence the outcome of the infection in a population of animals and its possible interaction with new horizontal infections involving the sexual cycle of the parasite in dogs. Phylogenetic analysis of the samples from this study, complemented by other genotypes previously described from other locations in the world, shows that *N. caninum* parasites appear to be closely related according to its place of geographical origin. In addition, this study has shown that some markers have a greater discriminatory power for the analysis of *N. caninum* isolates but they need to be evaluated further for their stability.

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5 Final Conclusions

The present work shows pathogenicity features of two new *Neospora caninum* isolates from goats. Besides being correlated with clinical diseases in naturally infected animals, the isolates didn't present a high pathogenicity level in an BALB/c mouse model, that was expected considering previous works that evaluated high and low pathogenicity isolates from dogs and cows. The genotyping of the goat isolates and clinical samples from goats and cows from the same region showed that the goat strains are closely related but were distant from the bovine strains. The use of new microsatellite markers suggested that isolates could be composed of multiple parasite strains and as they were multiplied in cell cultures, they could have lost this variability. Those results show the importance of the study of new *N. caninum isolates*, and give prospects to new studies to re-evaluate the knowledge that we have about pathogenicity evaluation of new strains.