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Chapter 1: Introduction to Neospora hughesi

Neospora, Sarcocystis and EPM

Neospora hughesi is a recently described cause of Equine Protozoal Myeloencephalitis (EPM), the most common neurological disease of horses in the US (Dubey et al. 1991, Fenger et al. 1997, Hamir et al. 1998, Dubey et al. 2000). Before the recognition of *N. hughesi* as a potential causative agent, all cases of EPM were attributed to *Sarcocystis neurona*. However, in a retrospective study of horses diagnosed with *S. neurona* EPM the organism could only be demonstrated in 67% by immunohistochemical staining of spinal tissues (Hamir et al. 1993). Serological and cerebrospinal fluid (CSF) tests used for the diagnosis of *S. neurona* EPM have been shown to cross react with *Neospora* antigens.

It is not currently known what proportion of EPM cases are caused by *Neospora*. The potential for a false diagnosis of *S. neurona* in a case of *Neospora* EPM is great due to antigen cross reactivity between the two parasites in western blot (WB) analysis. The WB is a commonly used test for the diagnosis of *S. neurona* EPM. The high prevalence of *S. neurona* antibodies in horses, approximately 50% in the U.S. and possible blood contamination of CSF samples also contributes to the potential of a false diagnosis (Dubey et al. 2000). *Neospora* is not widely recognized as an agent of EPM and is not often considered during the diagnostic process. Indeed in the few cases in which *Neospora* has been identified as the agent of EPM, diagnosis was made by post mortem immunohistochemical staining of tissue sections and not by the common screening procedures for EPM.

Differences between *N. hughesi* and *N. caninum*

Neospora caninum tachyzoites and cysts containing bradyzoites were first observed in dog tissues (Bjerkås et al. 1984; Dubey et al. 1988a, 1988b; Bjerkås and Presthus 1988). Oocysts were recently isolated from dog feces (McAllister et al., 1998; Lindsay et al. 1999). Dogs are the only known definitive host of *N. caninum*, although other canids are suspected to excrete oocysts. *Neospora caninum* has been shown to cause abortion, stillbirth, and congenital neurologic disease in dogs, cattle (Barr et al. 1991), cats (Dubey et al. 1990), deer (Woods et al. 1996), goats (Barr et al. 1992; Dubey et al. 1992), sheep (Dubey et al. 1992) and in experimental infections in mice (Cole et al. 1995). It was originally thought that the *Neospora* organism observed in all cases of equine neosporosis was *N. caninum* (Dubey and Porterfield 1990; Gray et al. 1996, Marsh et al. 1996, Lindsay et al. 1996, Daft et al. 1997), but it has since been identified in at least three cases as a new species called *N. hughesi* (Marsh et al.1998, Hamir et al. 1998, Cheatle et al. 1999).

Prior to the description by Marsh et al. (1998), the *Neospora* organisms observed in horses had never been cultured or characterized by molecular techniques. *N. hughesi* is morphologically very similar to *N. caninum*. The two can not be distinguished by light microscopy. However, ultrastructural examination by transmission electron microscopy (TEM) demonstrates a greater number of polar rhoptries in *N. hughesi* tachyzoites and a thinner tissue

cyst wall around *N. hughesi* bradyzoite stages. Differences in antigens were illustrated by western blot analysis and several differences were discovered in the Internal Transcribed Spacer (ITS-1) unit gene using PCR primers for *N. caninum* and sequencing the amplicon (Marsh et al. 1998). Differences have also been observed in the SAG1 and SRS2 proteins and the genes encoding them (Marsh et al. 1999). Additionally, specific reactivity with monoclonal antibody 6C11 raised against *N. caninum* antigen SAG1, which does not cross-react with *N. hughesi* SAG1 can be used to distinguish the two species (Marsh et al. 1999).

The serological techniques most commonly used to diagnose neosporosis are indirect fluorescent antibody tests (IFAT) and agglutination tests, which rely on serum antibodies to *N. caninum.* They can not distinguish between the species of *Neospora*. Only by scrutinizing a western blot for differences in antigen presentation can the species of *Neospora* infecting a live animal be determined.

It is probable that cases of *N. hughesi* in animals other than horses have been diagnosed as *N. caninum*. The prevalence of *N. hughesi* in other animals is not known; in order to understand the distribution of this parasite more techniques are needed that will help to distinguish between the two species of *Neospora*. Horses showing signs of EPM need to be tested for *Neospora* as well as *Sarcocystis* using a less cumbersome method than western blot to facilitate diagnosis of *Neospora* EPM.

Research Objectives

Research is needed before the veterinary community can adequately respond to this newly described cause of EPM. It is assumed, but not yet clearly demonstrated, that *N. hughesi* is very similar to *N. caninum* in its pathogenicity, transmission, and response to therapies. An experimental model is badly needed to test the efficacy of therapies against this new pathogen. The definitive host of *N. hughesi* must be identified in order to understand the life cycle and transmission of this agent and to properly advise horse owners about preventative management practices. Simple diagnostic tests to distinguish between the two *Neospora* species are badly needed in order to develop a clear understanding of the distribution of these two disease causing parasites. A determination of the antigenic similarity of the two species of *Neospora* would be helpful in ascertaining whether vaccines against *N. caninum* could be used to protect against *N. hughesi* neosporosis.

The purposes of these investigations are 1) to develop an experimental rodent model for disease caused by *N. hughesi* which could be used for testing potential therapeutic agents and for investigating the pathogenicity and immunology of *N. hughesi* neosporosis, 2) to determine whether the dog is the definitive host of *N. hughesi* and 3) to asses the genetic and antigenic similarity between the dense granule proteins of *N. caninum*, NcGRA7 and NcGRA6, and the homologous genes and proteins of *N. hughesi.*

Chapter 2: Review of pertinent literature

Case reports of *Neospora* in Horses

EPM is characterized by the classic symptoms of neurologic disease: head tilting, ataxia, circling, disorientation and muscle atrophy, but has also been associated with neurologic dysfunction in any form (Dubey et al. 1991). Horses of any age can be affected. EPM has been reported in North and South America, and in North America it is considered to be the most common equine neurologic disease (Dubey et al. 1991, MacKay et al. 1992, Hamir et al. 1998, Fenger et al. 1997). *Sarcocystis neurona* was formerly the only recognized cause of EPM, and the limited geographic range of the opossum, its definitive host, explained the range of the disease (Dubey et al. 1991). It is unclear why there have been no reports of *Neospora* induced EPM outside of the Americas. Details of equine cases of neosporosis currently in the literature are presented below.

Case 1: In 1990 Dubey and Porterfield reported finding *N. caninum* tachyzoites in the lung of an aborted equine fetus from North Carolina. The organism was 3-5 μm x 2-3 μm and divided by endodyogeny, which distinguished it from a *Sarcocystis* sp. The parasite reacted to anti-*N. caninum* serum in an avidin-biotin complex immunohistochemical staining. The report by Dubey and Porterfield (1990) was the first report of *Neospora* infection in a horse, and indicated that *Neospora* may be transmitted transplacentally in horses, possibly causing abortion. No other species of *Neospora* had yet been described, therefore no tests were available to distinguish the species of *Neospora* found in the equine fetus. It is not known whether *N. hughesi* is transmitted transplacentally, but the evidence indicates that it is a possibility.

Case 2: Visceral neosporosis was described in a 10-year-old Appaloosa mare with a history of weight loss and anemia (Gray et al. 1996). Lesions were found in the mesenteric lymph nodes and small intestine of the mare. Tachyzoite stages were seen in the lamina propria and submucosa of the small intestine and reacted to immunohistochemical staining for *N. caninum*. Some inflammation was observed in the colon. Enteritis led to malabsorbtion and weight loss. Sinuses of all mesenteric lymph nodes were filled with blood and aggregates of macrophages and neutrophils. The location of lesions indicated a recent oral infection that may have been advanced by the administration of large doses of the immunosuppressant corticosteriod drug dexamthasone for the four days preceding death. The drug was administered because of a history of chronic weight loss and anemia of unknown etiology that had not resolved. Only the tachyzoite stage of a *Neospora* species was observed in this horse. The case is the only case of *Neospora* associated enteritis. The horse had a history of unthriftyness and was most likely immunosuppressed, which would allow for more rapid and severe progression of disease, as well as the unusual manifestation.

Case 3: A 19-year-old Pinto mare with a 1-day history of rear limb paralysis and abnormal behavior was diagnosed after necropsy with neosporosis by Daft et al. (1996). Tachyzoites were observed by immunohistochemical staining in the brain, spinal cord, and peripheral nerves. Bradyzoite filled tissue cysts were observed in sections of the peripheral nerves indicating that

the infection was not recent. The spinal cord of the mare was focally hemorrhagic and there was a compression fracture. The mare also showed signs of Cushings disease which may have immunosuppressed the animal allowing for the reactivation of a latent *Neospora* infection.

Case 4: Neonatal neosporosis was described in a 1-month-old Quarter horse foal from Wisconsin (Lindsay et al. 1996). The thick-walled tissue cysts of *Neospora* were found in the thalamus, hypothalamus, brain and muscles around the eye of the foal. Parasites in these organs reacted to immunohistochemical staining for *N. caninum*. Lesions, but not parasites, were observed in the spleen, liver, mesenteric lymph nodes and heart. The foal had exhibited signs of visual and neurological problems since birth and the infection was assumed to be congenital.

Case 5: In 1996, Marsh et al. described *Neospora* in an 11-year-old Quarter horse in California which had exhibited mild incoordination for 3 months and suddenly developed incontinence and severe incoordination. The horse was diagnosed with *S. neurona* by Western blot of cerebral spinal fluid (CSF) performed by a commercial diagnostic laboratory. Upon microscopic examination of brain and spinal cord tissues, the organism was identified as *Neospora*. When the CSF blot was repeated with *S. neurona* and *N. caninum* whole tachyzoite antigen preparations side by side, only two reaction bands on the *S. neurona* blot corresponded to bands recognized by *S. neurona* positive control horses. A greater number of strongly reactive bands in the *N. caninum* blot corresponded to those of the *N. caninum* positive control. This indicated CSF antibody production to *Neospora* species and cross reactivity of these antibodies to *S. neurona*. Serum from the horse also reacted with both *S. neurona* and *N. caninum*, but with a more intense reaction to *S. neurona* antigens as indicated by darker bands. These results show that the horse might have been exposed to both agents, and that both, or just one may have been the cause of EPM in this case.

Case 6: A *Neospora* organism was reported in 1998 by Hamir et al. (1998) in the brain and spinal cord of a 20-year-old horse from Oregon with severe ataxia. The horse also had a thyroid adenoma and had been treated with dexamethasone once the diagnosis of EPM had been made in order to increase the number of protozoa. The horse tested positive for *S. neurona* by Western blot analysis of CSF, but not by PCR of CSF using primers specific for *S. neurona*. Microscopy and immunohistochemical analysis revealed large groups of *Neospora* tachyzoites associated with mild areas of inflammation. These results indicate again that there is cross reactivity with antibodies against *Neospora* species to some antigens of *S. neurona*.

Case 7: A 13-year-old Palomino Quarter horse with *Neospora* induced EPM was reported by Cheadle et al. in 1999. The horse presented with ataxia and rear limb weakness of five months duration. Western blot analysis of CSF was positive for the presence of antibodies to *Neospora* but not to *S. neurona*. Upon necropsy, no lesions were observed. Parasites were retrieved from cell cultures inoculated with homogenated sections of the spinal cord that corresponded to the rear limb weakness exhibited by the horse. The parasites divided by endodyogeny and reacted to known positive *N. caninum* test sera. The parasite was identified as *N. hughesi* based on Western blot profile and lack of reaction with monoclonal antibody 6C11 specific for the *N. caninum* SAG1 antigen.

Neospora hughesi as a new species

In 1998, Marsh et al. published a detailed description of the *Neospora* isolated and cultured from a neurologically impaired Quarter horse. They named the new species *N. hughesi* (Marsh et al. 1998). Morphological differences between *N. hughesi* and *N. caninum* are subtle and may be easily missed by light microscopy. Ultrastructural distinctions were made in the number of rhoptries: 13-27 in *N. hughesi*, 8-18 in *N. caninum*. The thickness of the tissue cyst wall also differs: 0.43 μm for *N. hughesi*, 0.15-1.0 μm for *N. caninum*.

Most reports of *Neospora* in horses appeared before the description of *N. hughesi*, thus the *Neospora* organism in these cases were assumed to be *N. caninum*, and a retrospective species distinction has not been made in most of those cases. Cross reactivity occurs between the two species of *Neospora* in most diagnostic procedures. Several differences in antigen presentation exist between *N. hughesi* and *N. caninum*, which make identification possible at the species level. Three antigens unique to *N. hughesi* of approximately 63, 29, and 16 kDa have been observed, and one antigenic protein of approximately 45 kDa of *N. caninum* does not seem to be expressed by *N. hughesi* (Marsh et al. 1998). Monoclonal antibody 6C11 raised to *N. caninum* antigen SAG1does not react with *N. hughesi* SAG1antigen (Marsh et al. 1999).

Comparisons of the Internal Transcribed Spacer region gene of all known isolates of *N. caninum* and of *N. hughesi* revealed consistent sequence differences at 7 nucleotide positions between the two groups (Marsh et al. 1998). Genetic and amino acid differences have also been reported in the genes for the SAG1 and SRS2 antigens of the two *Neosporas*. There is a 6.0% amino acid identity difference for SAG1 and 9.0% identity difference for SRS2 between the two species of *Neospora* (Marsh et al. 1999).

The case reports of neosporosis in horses reveal important aspects of EPM diagnosis. Cross reactivity of antibodies against *Neospora* species with *S. neurona* antigens may lead to false positive results. The presence of serum or CSF antibodies to *S. neurona* may indicate previous infection, and *S. neurona* may not be the disease causing agent. More than 50% of horses in North America have serum antibodies to *S. neurona*, and very few of these ever show signs of EPM (Hamir et al. 1998). Contamination of CSF samples either during sample collection or due to blood leakage into the CSF could lead to a false positive diagnosis. In a retrospective immunohistochemical study performed at the College of Veterinary Medicine at Oregon State University, *S. neurona* organisms were present in brain and spinal tissues of only 67% of horses diagnosed with EPM (Hamir et al. 1998). Another 30% of EPM horses that had been diagnosed with *S. neurona* did not have any detectable *S. neurona* stages in the spinal tissues. These results suggest that in up to 30% of western blot diagnosed *S. neurona* EPM cases the parasite can not be demonstrated; these may be false positive diagnoses. Diagnostic tests demonstrating antibody activity against *S. neurona* in the CSF of horses presenting with EPM, may mask cases of *N. hughesi* EPM.

The recognition of *Neospora* as an agent of EPM has been recent and the event is still considered rare, most EPM horses are tested only for *S. neurona*. Simple techniques to differentiate between *N. caninum* and *N. hughesi* have not been described, thus no survey comparing the prevalence of the two species has yet been reported. Dubey et al. (1999c)

reported that 69 of 296 (23%) horses from the United States had antibodies to *Neospora* spp. tachyzoites using the *Neospora* agglutination test (NAT). A survey of horses in Alabama, USA showed 12% prevalence of IgG antibodies to *Neospora* in asymptomatic horses by the indirect fluorescent antibody (IFA) test (Cheadle et al. 1999). This degree of exposure of U.S. horses to an agent with the potential to cause severe disease warrants further research.

Antibodies to *Neospora sp.* were not detected in 76 horses from Argentina (Dubey et al. 1999a) or 101 horses from Brazil (Dubey et al. 1999b) using the NAT. No explanation has been proposed for the difference in prevalence between the two American continents. The difference may be due to different equine management practices or to geographic limitation of the definitive host of *N. hughesi.*

Experimental *N. caninum* infections in Mice and Gerbils

Rodent models have facilitated research on *N. caninum* and led to the description of chemotherapeutic methods, production of a vaccine, the discovery of the definitive host and improved understanding of the pathogenicity and immunology of *N. caninum* infections. Similar experimental animal models for *N. hughesi* would be useful for the same purposes. The methods described in this thesis are based on work already done with *N. caninum* in an effort to compare the biological and pathological activity of the two species of *Neospora*.

There are three standard rodent models for *N. caninum* infection: the methylprednisolone acetate (MPA) immunosuppressed outbred mouse, BALB/c mouse, and gerbil models. These models, as well as infections in C57BL/6 mice, are well documented and routinely used in our laboratory. Accounts of *N. caninum* infections in mice in the literature, and extensive experience in our own lab were used to compare the pathogenicity of infections with the two *Neospora* species. Infection of gamma interferon gene knock out (γ-INFKO) mice with *N. caninum* has not been as well documented.

Standards for inoculation technique of mice with *N. caninum* have been most thoroughly examined by Lindsay and Dubey (1989, 1990). When modes of administration were compared, intraperitoneal (i.p.) infection with tachyzoites was the most consistent and caused death most rapidly. However, when mice were infected in this manner, death occurred before clinical signs of neosporosis were observable. Subcutaneous and oral inoculations with tachyzoites created more slowly progressing disease conditions. Bradyzoites were infective by subcutaneous or oral inoculation routes and created disease conditions similar to tachyzoites but the number of parasites administered to each animal was difficult to standardize because of the focal nature of bradyzoite aggregates (cysts). Only tachyzoites can be maintained in tissue culture. In the experiments described in this thesis, subcutaneous injection of tachyzoites was used to ensure infection with the desired concentration of *Neospora* organisms as it was found to be the most consistent mode of inoculation for the generation of lesions.

Using outbred Swiss Webster mice, Lindsay and Dubey (1989) found that when immunosuppressed with 4 mg of methylprednisolone (MPA) by intramuscular injection and infected with $2x10^5$ tachyzoites of *N. caninum*, the mice developed severe neosporosis with pneumonia, polymyositis, encephalitis, hepatitis, and pancreatitis. These mice died within 13

days post infection (PI). Swiss Webster mice given 2mg of MPA and the same tachyzoite load developed head tilting and impaired movement at 14 days PI. These mice developed mild chronic neosporosis. Encephalitis, pneumonia and polymyositis were evident at necropsy. Tissue cysts were found in the brains of these mice. Swiss Webster mice which were not immunosuppressed did not develop disease, nor were parasites observed in their tissues, but they did produce antibodies to *N. caninum*. The immunosuppressed outbred mouse model for acute or chronic neosporosis has been used extensively for research on *N. caninum* (Lindsay and Dubey, 1989, 1990a, 1990b, 1990c, Lindsay et al. 1991).

Lindsay et al. (1995) infected inbred BALB/c mice with *N. caninum* and found that CNS disease could be induced in these mice without the use of MPA. Infection of BALB/c mice with $2x10^5$ tachyzoites of the NC-1 strain of *N. caninum* caused CNS disease marked by head tilting and rear limb paralysis or weakness and lead to death within 26-70 days. Brain lesions were scattered with no regional preference, tachyzoites were seen in some lesions but no *N. caninum* cysts were demonstrated. BALB/c mice infected with the NC-3 strain of *N. caninum* did not develop clinical signs and showed only rare brain lesions of moderate intensity. The BALB/c rodent model is more useful than the immunosuppressed mouse model for testing therapies against neosporosis because of the observable onset of signs and the extended duration of disease, which allows time for intervention and facilitates evaluation of treatment efficacy. The BALB/c mice are less likely to succumb to opportunistic infections than are immunosuppressed mice.

Central nervous system disease can also be induced in C57BL/6 mice without MPA when given a high dose of $5x10^7$ tachyzoites, of NC-1 strain *N. caninum* (Long et al. 1998). Lesions seen in these mice were similar to those seen in BALB/c mice at all levels of infection. In a study comparing the pathogenicity of the NC-1 strain of *N. caninum* in C57BL/6 and BALB/c mice, no significant differences were observed. In both strains, early infection was marked by nonfatal pulmonary granuloma formation and hepatic necrosis not associated with mortality. Death due to encephalitis occurred only in the groups receiving the highest dose of $5x10^7$ tachyzoites, and occurred most often after the sixth week of infection (Long et al. 1998). In contrast, Eperon et al. (1999) found that wild type C57BL/6 mice infected intraperitoneally with 10^5 tachyzoites of the NC-1 strain were resistant to neosporosis but B-cell deficient C57BL/6 mice died within 29 days when administered the same dose. The difference in these results of *N. caninum* infection in C57BL/6 mice may be due to decreased parasite virulence that occurs over time when the organism is continually passaged through cell cultures, or to an inconsistent response by C57BL/6 mice to *N. caninum*. Therefore the C57BL/6 mouse is not used as a model for *N. caninum* infection.

Interferon gamma gene knockout (γ-INFKO) mice are not used as a model for *N. caninum* infection because mortality occurs too rapidly and CNS signs are not present. In these mice *N. caninum* infection causes severe damage to the liver and spleen and often the lungs. The γ-INFKO mice are used as a bioassay for the presence of *N. caninum* oocysts.

Gerbils are susceptible to infection with either tachyzoites or oocysts of *N. caninum* (Gondim et al. 1999, Dubey and Lindsay 2000). *Neospora caninum* tachyzoites can be retrieved from the tissues of gerbils when infected intraperitoneally (Gondim et al. 1999). Gerbils fed 10

oocysts showed lesions in the brain and significant seroconversion, and gerbils fed ~1000 oocysts died within 6-13 days (Dubey and Lindsay, 2000). Gerbils were proposed as an advantageous alternative to γ-INFKO mice as a bioassay for the detection of *N. caninum* oocysts in dog feces because of their high sensitivity to few oocysts and lower cost.

Dogs as the definitive host of *N. caninum*

The dog was shown to be the definitive host of *N. caninum* by McAllister et al. in 1998. *Neospora* tissue cysts of the NC-2, and NC-beef strains were produced in immunosuppressed outbred white mice. Brains of the mice were harvested at 1 month PI and fed to 8-week-old beagles. Oocysts were observed between 8 and 27 days after infection of the dogs, in a sucrose flotation procedure. Oocysts were placed in 2% H_2SO_4 and aerated on a shaker for 3-7 days and then inoculated orally and subcutaneously into mice. Tachyzoites were observed in these mice, and were identified as *N. caninum* in a PCR assay using *N. caninum* specific primers. Lindsay et al. (1999) recently confirmed these results using the NC-Liverpool and NC-beef strains of *N. caninum*.

Dogs do not appear to be an optimal definitive host for *N. caninum* because the infection rate is low and few oocysts are produced (McAllister et al. 1998). *Neospora caninum* can be perpetuated in cattle by repeated congenital transmission to healthy offspring**.** However, the prevalence of antibodies to *Neospora* in a wide variety of herbivorous and carnivorous mammals in which repeated congenital transmission has been documented indicates that there is also an environmental source of *Neospora* infection**.** Lindsay et al. (2000) have proposed that another canid may be a better definitive host.

The Dense Granule Proteins

Neospora caninum tachyzoites adhere to and induce the host cell membrane to invaginate and bud off within the host cell (Hemphill et al. 1995). The result is a protective vacuole, called the parasitophorous vacuole that does not acidify or fuse with lysosomes. Within the parasitophorous vacuole the parasite undergoes multiple bilateral asexual reproduction called endodyogeny. Several secretory organelles, the rhopteries, micronemes and dense granules, produce compounds that modify the vacuole membrane and lumen (Cesbron-Delauw 1994). Compounds secreted by these organelles are released upon host cell rupture due to parasite infection and they may be excreted by infected host cells (Fischer et al. 1998). Mice immunized with purified *T. gondii* dense granule proteins GRA2 or GRA5 are protected against lethal infection with the parasite (Darcey et al. 1992). Mice immunized with *N. caninum* dense granule protein GRA7 are protected against lethal infection (Lindsay personal communication 1999).

Dense granule organelles are found in the bodies of all apicomplexan parasites, their secreted proteins (GRA proteins) are released continually after host cell penetration and are targeted to the parasitophorous vacuole membrane or to the vacuolar space. The dense granule proteins are thought to play an important role in intracellular survival, nutrient uptake and waste excretion (Cesbron-Delauw 1994). As is the case with many of the antigenic proteins of *N. caninum*, the dense granule proteins share a significant homology, both at the genetic and amino

acid level, with those of *T. gondii* and so are prefixed with "Nc" and then given the name of their nearest *T. gondii* homologue (Howe et al. 1999).

The two dense granule proteins examined in this research, NcGRA6 (syn NCDG2) and NcGRA7 (syn NCDG1), are antigenic dense granule proteins unique to *N. caninum* which were isolated by screening an *E. coli* cDNA library of *N. caninum* genes (Hemphill et al. 1998, Liddell et al. 1998). Characterization of the genes encoding these proteins has been completed (Genebank access numbers AW119252, and AF029350).

Neospora caninum GRA7 shares a 42% protein homology with *T. gondii* GRA7 (Jacobs et al. 1997) and should be called NcGRA7 (Howe et al. 1999). In *T. gondii* infected cells, TgGRA7 is associated with the PV membrane and can be detected extending into the host cell cytoplasm (Jacobs et al. 1997). TgGRA7 has also been detected in the supernatant of infected cells and is thought to be an indicator of intracellular infection with *T. gondii* (Fischer et al. 1998). NcGRA7 is thought to play a role in penetration of the host cell and maintenance of the parasitophorous vacuole membrane and is expressed by both the tachyzoite and bradyzoite stages of *N. caninum* (Hemphill et al. 1998).

NCDG2 shares 36% amino acid homology with *T. gondii* GRA6 and is referred to as NcGRA6 (Howe et al. 1999). Both the *T. gondii* and the *N. caninum* GRA6 have a hydrophobic region that is likely a transmembrane domain suggesting that the protein is associated with and may span the PV network membranes (Lecordier et al. 1995, Liddell et al. 1998).

Recombinant forms of NcGRA6 and NcGRA7 are the antigen used for the rELISA diagnostic procedure for *N. caninum* infection (Lally et al. 1996). Cattle experimentally infected with *N. caninum* had high antibody titers to both antigens at 54 days PI and returned to preinfection levels within 103 days PI. When sera were tested for reactions with each antigen separately, titers to NcGRA6 were low when titers to NcGRA7 were high and vice versa. This indicates that the two antigens may be released at separate times by the parasite, or that they exit the host cell at separate times in infection. No cross reactivity was observed when sera from cattle infected with *T. gondii* oocysts or *S. neurona* sporocysts were tested.

The GRA6 and GRA7 genes and proteins of *N. hughesi* have not previously been characterized. Differences in these antigens may serve as the basis of diagnostic serology to distinguish between the two species of *Neospora* and better understand the distribution of these two parasites. Homology between the GRA proteins of the two *Neospora* species, especially the GRA7 protein which has been shown to be protective in mice, may lead to the production of a vaccine which would protect against both pathogens.

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Chapter 3 of this thesis has been accepted for publication in Veterinary Parasitology.

Specific contributions of each of the authors are as follows:

Catherine P. Walsh: Performed all experiments. Wrote majority of paper text.

Anne M. Zajac: Editing and advice on serology results.

Robert B. Duncan: Histology descriptions and photography.

David S. Lindsay: Provided all materials, assisted in planning and performance of all experimental procedures, editing and contributing to text.

B.L. Blagburn: Provided initial stock of *N. hughesi* Nh-A1 strain.

Chapter 3: Neospora hughesi: *Experimental infections in mice, gerbils, and dogs*

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Abstract

Neospora hughesi is a recently described cause of equine protozoal myeloencephalitis (EPM). A rodent model for pathogenicity would facilitate development of therapies to be used in horses. In the present study, we examined the susceptibility of BALB/c γ-interferon gene knockout (γ-INFKO), BALB/c, CD-1, and C57BL/6 strains of mice and gerbils to infection with tachyzoites of the Nh-A1 strain of *N. hughesi* isolated from a horse from Alabama, USA. Only the γ-IFNKO mice developed severe clinical disease following infection with *N. hughesi* and died 19-25 days after infection and exhibited severe cardiac lesions. In contrast, experimental infection of γ-INFKO mice with tachyzoites of the NC-1 or NC-Liverpool strains of *N. caninum* resulted in deaths 8-10 days after infection. The most severe lesions were in the livers, spleens, and lungs of these mice. Gerbils inoculated with *N. hughesi* did not develop clinical disease, had few microscopic lesions but did seroconvert. Two dogs fed the brains of mice, shown to contain *N. hughesi* tissue stages by cell culture and γ-IFNKO mouse bioassay, did not shed *N. hughesi* oocysts over a 23 day observation period. The marked difference in pathogenicity between the 2 species of *Neospora* in γ-IFNKO mice, and lack of oocyst excretion by dogs fed *N. hughesi* infected mice are additional evidence that the species distinction between *N. caninum* and *N. hughesi* is valid.

Key words: Neospora hughesi; *Neospora caninum*, Mouse; Gerbil; Dog; Horse; Model

1. Introduction

Neospora hughesi is a recently described apicomplexan parasite that is a cause of equine protozoal myeloencephalitis (EPM) (Marsh et al., 1998). Before 1998, *Neospora* organisms had been reported in horses on several occasions (Dubey and Porterfield 1990; Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996; Daft et al., 1997; Hamir et al., 1998). Clinical signs in these cases were variable and included anemia, blindness, weight loss, rear limb paralysis, ataxia and abortion. The *Neospora* organisms were found most often in the brain and spinal cord, but in some cases were also found in the peripheral nerves, muscles of the eye, intestine or the aborted fetus. Marsh et al. (1998) and Cheadle et al. (1999) have successfully isolated and cultured *N. hughesi* from horses with EPM.

Neospora hughesi is morphologically similar to, but ultrastructurally, antigenically, and genetically distinct from the only the other member of the genus: *N. caninum (*Marsh et al., 1998; 1999). Little is known about the life cycle or prevalence of *N. hughesi*. Dubey et al. (1999c) reported that 69 of 296 (23%) horses from the United States had antibodies to *Neospora* sp. tachyzoites using the *Neospora* agglutination test (NAT). Cheadle et al. (1999) found that 62 of 536 (12%) horses from Alabama, USA had antibodies to *Neospora sp.* tachyzoites by the indirect fluorescent antibody (IFA) test. Antibodies to *Neospora sp.* were not detected in 76 horses from Argentina (Dubey et al., 1999a) or 101 horses from Brazil (Dubey et al., 1999b) using the NAT.

The present study was done to determine the susceptibility of mice and gerbils to infection with tachyzoites of the Nh-A1 strain of *N. hughesi* . Additionally, dogs were fed *N. hughesi* infected mouse brains to determine if they would excrete oocysts in their feces as they have been reported to do after consuming *N. caninum* tissue cysts (McAllister et al., 1998; Lindsay et al., 1999).

2. Materials and Methods

2.1. Cell culture and parasite inoculum preparation

Tachyzoites of the Nh-A1 strain of *N. hughesi* (Cheadle et al., 1999) were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, Maryland, USA). The CV-1 cells were grown to confluence in 75 cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 25 mM HEPES buffer, 100 U penicillin Gml^{-1}, and 100 streptomycin $mgml^{-1}$. Cell cultures were maintained in growth medium in which the FBS content was lowered to 2%. Cell cultures were incubated at 37°C. For comparative pathogenicity studies, tachyzoites of the NC-Liverpool strain of *N. caninum* (Barber et al., 1997) and NC-1 strain of *N. caninum* (Dubey et al., 1988) were grown in CV-1 cells using identical procedures.

For quantitative studies, tachyzoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and

magnesium (HBSS). The CV-1 cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10-ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 μm filter to remove cellular debris. The numbers of tachyzoites in the filtrate was determined using a hemocytometer. The final volume of suspension was adjusted to the desired concentration of tachyzoites present for inoculation.

For general maintenance of tachyzoites, monolayers were examined with an inverted microscope for the development of either lesions (areas devoid of host cells caused by parasite replication) or the presence of many extracellular tachyzoites. Once lesions were observed or many extracellular parasites were present, the monolayer was scraped with a cell scraper and 5 ml of the tachyzoite containing fluid was transferred to 2 flasks of CV-1 cells. Tachyzoites of *N. hughesi* were passaged in this manner every 3 to 7 days.

2.2 Neospora agglutination test (NAT)

Tachyzoites of the NC-1 strain of *N. caninum* were used as antigen for the NAT (Packham et al., 1998). Tachyzoites were collected from infected cell cultures as described above and fixed in 2 ml of 37% formaldehyde solution which was then diluted to 6% with phosphate buffered saline (PBS) and stored at 4°C. Prior to use in the direct agglutination test the tachyzoites were washed twice in PBS and resuspended in alkaline buffer containing eosin at 4 x 10^7 tachyzoites per ml^{-1}. The eosin aids in the visualization of the agglutination reaction. The direct agglutination test was conducted in 96 well, round bottom (U bottom) plates. Test sera were diluted with PBS and 25 μl of serial test dilutions is added to 25 μl of 0.2 M 2-mercaptoethanol. This was done to destroy all IgM antibodies and prevent non-specific agglutination caused by IgM molecules. This solution was combined with 50 μl of antigen solution and mixed thoroughly by pipetting up and down several times. Positive and negative control sera were run on each plate. The plates were covered and incubated overnight at 37°C in a CO_2 incubator. The reaction was read the next morning. Diffuse white opacity across the entire diameter of the well was considered a positive agglutination reaction. A central discrete opaque dot or button was considered a negative reaction.

2.3. Experimental inoculations of mice and gerbils

All mice and gerbils used were females and all inoculations were given subcutaneously unless specifically noted. Mice used were BALB/c γ-interferon gene knockout (γ-IFNKO), BALB/c, CD-1, and C57BL/6 mice from commercial suppliers. Mice and gerbils were housed in groups of 4 or 5 in plastic box cages with water and rodent chow provided *ad libitum*. All animals in this study were cared for in accordance with the Virginia Polytechnic Institute Animal treatment guidelines and Good Laboratory Procedure policies.

Table 1 summarizes the different strains of mice, experimental treatments, numbers and results. The γ-IFNKO, BALB/c, CD-1, and C57BL/6 mice and gerbils were inoculated with either 5 x 10^4, 2 x 10^5, or 5 x 10^5, tachyzoites of *N. hughesi*. Two groups of CD-1 mice were immunosuppressed with either 2 or 4 mg of methylprednesalone acetate (MPA) on days –7, 0, and 7 post inoculation (PI) as described previously (Lindsay and Dubey, 1989). Control groups

of BALB/c, CD-1, and C57BL/6 mice were inoculated with an equal volume of Hanks balanced salt solution (HBSS). A group of γ-IFNKO mice was inoculated with 1 x 10^5 tachyzoites of the NC-Liverpool strain *N. caninum* and a group of γ-IFNKO mice was inoculated with 5 x 10^4 tachyzoites of the NC-1 strain of N. *caninum*. All mice were bled and killed by CO_2 asphyxiation at 8 weeks post infection (PI) with the exception of BALB/c mice, which were bled and killed at 17 weeks PI. Gerbils were bled and killed at 7 weeks PI.

Mouse strain	Dose of N. hughesi tachyzoites	Dose MPA	Range of titers	Number of mice with Lesions/ Total Number of mice in group	Number of mice that died/Total number of mice in group
γ-IFNKO	5 x 10^4	NA	NA	5/5	5/5
γ-IFNKO	2 x10^5	NA	NA	4/4	4/4
CD-1	2 x 10^5	2mg	25-200	3/5	0/5
CD-1	2 x 10^5	4mg	25-50	4/4	0/4
CD-1	2 x 10^5	NA	25-200	3/5	0/5
CD-1	None	NA	<25	0/5	0/5
C57BL/6	2 x 10^5	NA	<25-50	0/5	0/5
C57BL/6	None	NA	<25	0/5	0/5
BALB/c	2 x 10^5	NA	<25-50	0/5	0/5
BALB/c	5 x 10^5	NA	25-50	1/5	0/5
BALB/c	None	NA	<25	0/5	0/5
GERBIL	5 x 10^5	NA	>800	2/4	0/4

Table 3.1: Protocol for inoculation of mice with *N. hughesi* tachyzoites, titer ranges and lesion prevalence.

Susceptibility to infection was evaluated by mortality, presence of lesions, and antibody titer to *N. caninum* tachyzoites as measured by the NAT test. Unfixed portions of brain from immunosuppressed CD-1 mice were suspended in HBSS, vortexed, passed through a 22-gauge needle and inoculated on to CV-1 cell cultures or into γ-IFNKO mice to assay for the presence of low numbers of parasite stages.

Samples of liver, lung, tongue, kidney, adrenal gland, eye, brain, thigh muscle and skin were collected from each mouse and fixed in 10% neutral buffered formalin, processed through graded ethanols, a xylene substitute, and embedded in paraffin. All animals found moribund were killed and samples collected. The fixed samples were processed for histological examination and stained with hematoxylin and eosin.

2.4. Inoculation and examination of dogs

Thirty CD-1 mice were divided into 6 cages of 5 mice each and were immunosuppressed with 2 mg MPA –7 days PI, 4 mg MPA on the day of infection, and 2 mg MPA 7 days PI. They were infected with 2.5 x10^5 tachyzoites of *N. hughesi*. Two mice from each of the 6 groups were bled on days 74 PI and 133 PI. All mice in the 6 groups were killed by CO_2 asphyxiation on day

133 PI and the brains removed. One mouse brain from each group (6 mice total) was homogenized by repeated passage through a 10 ml syringe with a 22 gauge needle. The homogenate from each mouse was divided in two and half used to infect CV-1 cell cultures the other half injected into 1 γ-IFNKO mouse. The remaining 24 mouse brains were fed whole to dogs.

Two mixed-breed littermate dogs about 15 weeks of age were used. Before use both dogs were negative (<1:25 titer) for antibodies to *N. caninum* as determined by IFA (Cole et al., 1995) and NAT, and to *Toxoplasma gondii* as determined in the modified direct agglutination test (Dubey and Desmonts, 1987).

Both dogs were given 100 mg MPA intramuscularly on days –7 and 0 post-feeding (PF) of infected mouse brains to potentially increase the susceptibility to intestinal infection. Each dog was fed 12 mouse brains. The brains were placed in a bowl that did not contain food and were consumed within 30 seconds by each dog. Blood was collected from each dog on days –14, -7, 0, 5, 10, 14, 19, and 27 days PF, the serum separated, and examined for IgG antibodies by the IFA test and NAT. Sera were examined at an initial dilution of 1:25 and then endpoint titrated by doubling dilutions. Fecal samples were collected on days –2, -1, 0, 3-23 PF and examined for oocysts after flotation with Sheather's sugar solution (Ernst and Benz, 1981).

All feces were collected on days 4-18 PF, soaked, and mixed in 2% sulfuric acid, strained through double layered cheese cloth and placed in an open toped Erlenmeyer flask on a slowly rotating shaker for 4 to 5 days to allow oocysts to sporulate. After shaking the samples were refrigerated for up to 6 weeks. Samples were processed for oocyst concentration, as previously described (Lindsay et al., 1999) and fed to 2 groups of 3 γ-IFNKO mice, the first group receiving material from days 4-11 PF and the second group receiving material from days 12-18 PF.

The dogs were euthanized by intravenous overdose of sodium pentobarbital on day 42 PF. Portions of tissues from brain, eyes, heart, tongue, lung, liver, spleen, kidney, adrenal gland, mesenteric lymph nodes, skeletal muscles, and intestines were collected, fixed in 10% neutral buffered formalin solution, and processed as described previously. Sections were cut and stained with hematoxylin and eosin.

3. Results

3.1 Mouse and gerbil infections

Only γ-IFNKO mice experienced mortality as a result of infection with *N. hughesi*. All γ-IFNKO mice inoculated with 5×10^4, or 2×10^5 tachyzoites died within 19 or 25 days of infection, respectively. These mice exhibited a rough hair coat, curled back, lethargy and loss of appetite. The CD-1 mice immunosuppressed with 4 mg of MPA exhibited mild signs consisting of rough hair coats at 17 days PI but recovered. None of the other groups of mice exhibited clinical signs.

In all γ-IFNKO mice inoculated with *N. hughesi* tachyzoites, lesions were in various organs including heart, brain, liver, lung, tongue, pancreas, thymus, spleen, and skeletal muscle. The most lesions severe were in the heart and could be seen grossly as large pale areas. Microscopically there was severe myocarditis, atrial thrombosis, fibroplasia, myocardial fiber

atrophy, myocardial fiber loss and infiltrates of macrophages, neutrophils and eosinophils (Fig. 1). Parasites were scarce in lesions but occasionally groups of tachyzoites were present (Fig. 1). Lesions in the brains were mild to moderate multifocal meningoencephalitis with perivascular infiltrates of neutrophils, macrophages, and eosinophils. Multifocal to focal areas of vascular congestion, hypertrophy of endothelial cells, gliosis, and spongiosis were also commonly observed in the brains of these mice (Fig. 2). Definitive tissue cysts were not identified in the brain or other tissues. Interstitial pneumonia of varying severity was present in the lungs. Small aggregates of neutrophils, eosinophils, and macrophages were present in the portal and centrolobular interstitum and in the hepatic sinusoids of the livers. The spleens had mild lymphoid follicular hyperplasia and the follicles had multifocal lymphoid necrosis. Lesions in the pancreas consisted of mild to moderate pancreatitis with interstitial infiltrates of macrophages, neutrophils and eosinophils with minimal to mild exocrine atrophy. Moderate lymphoid necrosis was observed in the thymus. Multifocal myocyte necrosis with infiltrates of macrophages, neutrophils and eosinophils were observed in skeletal muscles and tongue.

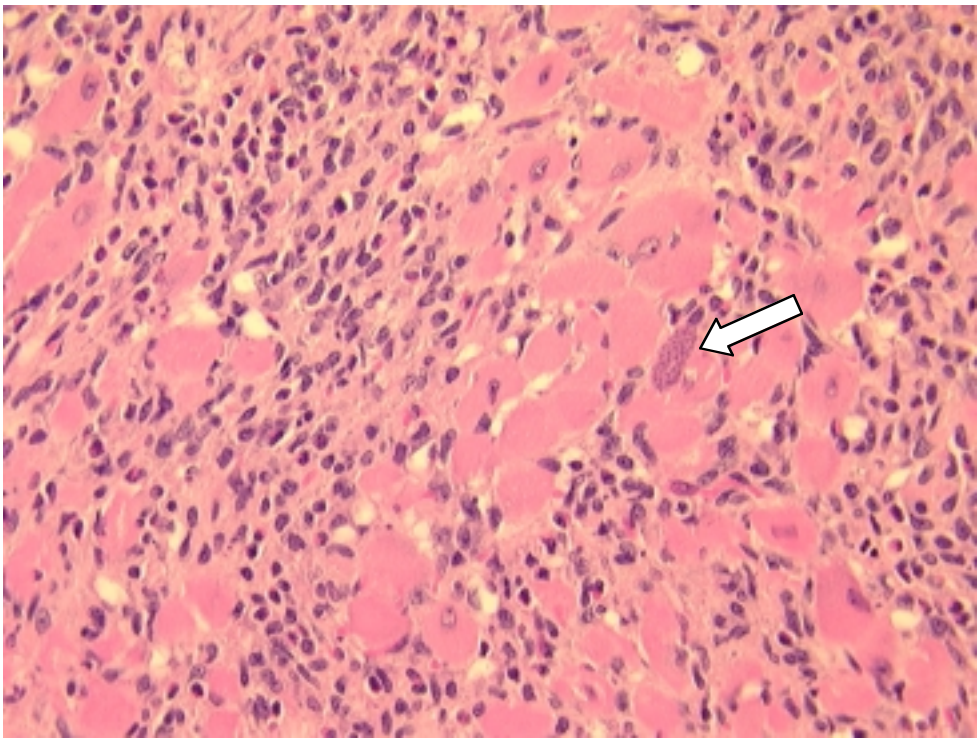

Figure 3.1: Heart from an γ-interferon gene knockout mouse infected with *Neospora hughesi* tachyzoites. A group of tachyzoites (arrow) is present and most of the myocardial fibers have been replaced by fibrous tissue.

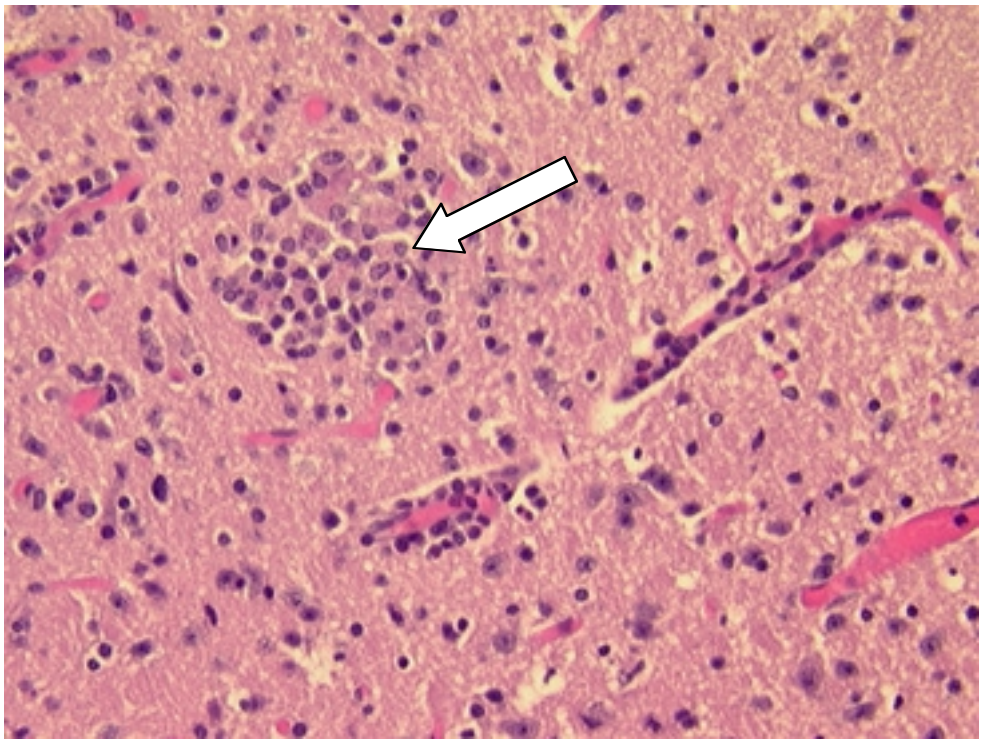

Figure 3.2: Brain from an γ-interferon gene knockout mouse infected with *Neospora hughesi* tachyzoites. An area of gliosis (arrow) is present.

No lesions were observed in BALB/c mice infected with 2 x10^5 tachyzoites. Only 1 BALB/c mouse in this treatment group had a titer of 1:25 by the NAT, the others had no titer (<1:25). Three of 5 BALB/c mice infected with 5 x 10^5 tachyzoites had lesions in their tissues. The lesions were in the livers and consisted of multifocal sinusoidal aggregates of lymphocytes in 2 mice, and subacute hepatocellular necrosis in 1 mouse. Four of 5 mice in this group had a titer of 1:25; 1 mouse had a titer of <1:25.

Of the CD-1 mice immunosuppressed with 2 mg MPA, 1 exhibited a minimal focus of interstitial lymphocytes in one portion of the heart. One other mouse showed marked granulopoiesis in the spleen and liver. These 2 mice and 1 other mouse had minimal perivascular cuffing in the brainstem and minor mixed cuffs in the cerebrum. The remaining 2 mice in this group had no lesions. The CD-1 mice in this group had titers of 1:200 (2 mice), 1:50 (2 mice) and 1:25 (1 mouse). All CD-1 mice immunosuppressed with 4 mg MPA had lymphoplasmocytic perivascular cuffs in the brain. Their other tissues were normal. Titers of mice in this group were 1:50 (2 mice) and 1:25 (2 mice). Of the non-immunosuppressed CD-1 mice, 3 of 5 had minor foci of lymphocytic sinusoidal inflammation in the liver, 1 also had lymphohistolytic inflammation of the epicardial fat and 2 had no lesions. The non-immunosuppressed CD-1 mice had titers of 1:200 (2 mice) and 1:25 (3 mice).

Cell cultures inoculated with the brains of both groups of immunosuppressed CD-1 mice demonstrated the presence of *N. hughesi* stages within 2 weeks of inoculation. All γ-IFNKO mice which were sub-inoculated with brain homogenates from the MPA suppressed CD-1 mice died within 42 days.

No C57BL/6 mice had lesions. Titers of C57BL/6 mice were 1:50 (1 mouse) 1:25 (2 mice) and <1:25 (2 mice).

None of the non-infected control BALB/c. CD-1, or C57BL/6 mice had lesions. All had titers of <1:25 in the NAT.

Two of 4 gerbils had lesions in the brain. One had mild lymphoplasmocytic focal meningoencephalitis and the other had minimal lymphoplasmocytic meningitis. All gerbils had small sinusoidal areas of chronic inflammation consisting of lymphocytes, plasma cells, macrophages, and rare multinucleated cells. No parasites were observed in the gerbils by histological examination, and gerbils exhibited no clinical signs. All gerbils had titers of ≥1:800 in the NAT.

The γ-IFNKO mice infected with 5 x10^4 NC-1strain or 1 x 10^5 NC-Liverpool strain tachyzoites of *N. caninum* died or were killed because of severe illness 8 to 10 days PI. No sera were collected from these mice. Lesions in these mice were found in multiple organs except the brain. They were most severe in the spleen and liver. Severe multifocal coalescing splenic necrosis associated with tachyzoites was observed in the spleen. Lesions in the liver consisted of multifocal coalescing hepatic necrosis associated with tachyzoites. Mild interstitial pneumonia was present in the lungs. Lesions in the hearts were small and rare and consisted of myocardial necrosis.

3.2 Infections of dogs

Cell cultures layered with brain homogenate from the mouse groups fed to the dogs were positive for *N. hughesi,* and γ-IFNKO mice inoculated with the homogenate died within 36 days. Neither dog excreted *Neospora*-like oocysts in its feces. On days 11-21 after infection *Isospora ohioensis*-like oocysts were observed in the feces of both dogs. No *Neospora* parasites were seen in the tissues of the 2 dogs. Microscopic lesions suggestive of neosporosis were not observed in either dog. No γ-IFNKO mice fed concentrated fecal material from the dogs died or had antibodies in the NAT.

4. Discussion

The present study provides information on the susceptibility of strains of mice and gerbils to *N. hughesi* neosporosis. It demonstrates that of the mouse strains investigated, only γ-IFNKO mice are susceptible to severe disease caused by *N. hughesi* and that the infection produced is markedly different from that caused by *N. caninum*. Lesions associated with *N. caninum* neosporosis in γ-IFNKO mice are most severe in the spleen, liver and lungs. In contrast, lesions associated with *N. hughesi* neosporosis in γ-IFNKO mice are most severe in the heart leading to atrophy of the muscular framework. The drastic difference in course of infection between the 2 *Neospora* species provides further validation of the distinction between them. We propose that because of its unique, obvious and consistent pathogenicity, the γ-IFNKO mouse model be used as a bioindicator of *N. hughesi* infection and for the testing of therapies to be used against *N. hughesi* neosporosis in horses.

Inbred BALB/c mice are susceptible to *N. caninum* neosporosis, especially to the NC-1 strain which causes multiple brain lesions and death (Lindsay et al., 1995; Baszler et al., 1999). Three of 5 BALB/c mice that received the high dose of tachyzoites developed mild multifocal lesions in the liver. No BALB/c mice that received the lower dose developed lesions. All BALB/c mouse titers were low. No BALB/c mice died in the 17 week experimental period. *Neospora hughesi* appears to be less pathogenic for BALB/c mice than is *N. caninum.*

Outbred mice are resistant to *N. caninum* neosporosis unless treated with MPA (Lindsay and Dubey, 1989, 1990a, 1990b; Lindsay et al., 1992). The CD-1 mice used in the present study were resistant to fatal neosporosis even when immunosuppressed with MPA. All CD-1 mice infected with *N. hughesi* developed a titer detectable by the NAT test. The MPA suppressed mouse model used for testing therapies against *N. caninum* neosporosis (Lindsay and Dubey, 1992a) would not be useful for *N. hughesi* because of the lack of pathogenicity.

Lesions were not observed in C57BL/6 mice and only 1 of 5 C57BL/6 mice developed a titer. This indicates that they are resistant to *N. hughesi* infection.

Gerbils are susceptible to infection with *N. caninum* tachyzoites (Cuddon et al., 1992; Gondim et al., 1999) and oocysts (Dubey and Lindsay, 2000). Gerbils infected with similar numbers of tachyzoites of the NC-1 strain of *N. caninum* may die within 14 days of infection (Lindsay unpublished). Gerbils in this study showed few minor brain and hepatic lesions caused by *N. hughesi* infection. All gerbils developed relatively high titers ($\geq$1:800) in the NAT. No gerbils died or showed clinical signs.

Dogs fed the brains of *N. hughesi* infected CD-1 mice did not shed *N. hughesi* oocysts detectable by fecal flotation and no γ-INFKO mice fed concentrated fecal material from these dogs died or developed a titer to *Neospora* species. This suggests that dogs are not the definitive host of *N. hughesi.* However, studies indicate that not all dogs will excrete *N. caninum* oocysts after being fed tissue cysts (McAllister et al., 1998). Additional studies in dogs need to be conducted to determine their role in the transmission of *N. hughesi*. It is interesting to note that equine neosporosis has only been observed in the Americas (Dubey and Porterfield 1990; Daft et al., 1997; Gray et al., 1996; Lindsay et al., 1996; Marsh et al., 1996; Hamir et al., 1998). This may indicate that the definitive host of *N. hughesi* is limited to the Americas.

Acknowledgments

This study was funded in part by a grant from the Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech.

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Chapter 4 of this thesis will be submitted to the Journal of Eukaryotic Microbiology.

Specific contributions of each of the authors are as follows:

Catherine P. Walsh: Performed all experiments, collected data, primary author.

Nammalwar Sriranganathan: Advised on molecular technique and strategy, assisted in editing.

Mark Jenkins: Provided the polyclonal monospecific antibodies against *N. caninum* GRA6 and GRA7.

Anne M. Zajac: Assisted with data interpretation and editing.

David S. Lindsay: Provided all materials, advised, assisted with editing.

Chapter 4: Differentiation of *Neospora hughesi* from *Neospora caninum* based on the dense granule proteins, GRA6 and GRA7

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Abstract

Neospora hughesi is a recently described apicomplexan parasite that has been associated with several cases of equine protozoal myeloencephalitis (EPM). The biology of this new parasite is just beginning to be defined. Towards this understanding, we report important differences between the nucleotide and deduced amino acid sequences of the dense granule proteins GRA6 and GRA7 of *N. hughesi* and *N. caninum*. This information can be used to differentiate the two species and contribute to further understanding of the prevalence and biology of *N. hughesi*. The newly defined proteins of *N. hughesi* are referred to as NhGRA6 and NhGRA7 in keeping with the protocol for naming homologous proteins of the apicomplexans. The deoxyrobonuclotide sequences of the two *N. hughesi* dense granule genes were determined. From analysis of the sequences we found that there is a 14.8% difference in deduced amino acid sequence between NhGRA7 and NcGRA7, and a 4% difference between NhGRA6 and NcGRA6 in areas that could be compared. The gene for NhGRA6 is interrupted by an insertion along with several nucleotide differences that result in a protein product that is 33 amino acids larger than NcGRA6 and a PCR amplicon that migrates visibly slower in a 1% agarose gel. This was further confirmed by western blot analysis in which NcGRA6 monospecific antibodies recognized a protein of approximately 42 kDa in *N. hughesi* whole tachyzoite preparation and a protein of 37 kDa in *N. caninum* whole tachyzoite preparation.

Introduction

Neospora hughesi is a newly recognized cause of equine protozoal myeloencephalitis (EPM). It is closely related to *Neospora caninum*, a parasite which causes central nervous system disease marked by ascending hind limb paralysis in the dog definitive host when transmitted transplacentally. When other mammals consume the oocysts of *N. caninum* they become the intermediate host and the infection can lead to abortion (Bjerkas et al. 1984, McAllistar et al. 1999, Lindsay et al. 1999). *Neospora hughesi* has been definitively isolated and identified on only three occasions, but may have been misidentified as *N. caninum* in the past, as there were few diagnostic techniques to differentiate between the species (Marsh et al. 1998, Hamir et al. 1998, Cheatle et al. 1999). The serological tests used to obtain the majority of prevalence data for *N. caninum*, are based on serum antibodies, and can not distinguish between the two *Neospora* species due to a high level of antigenic cross reactivity.

The two members of the genus can be distinguished by genetic differences in the ITS-1, SAG1 and SRS2 genes, and by differential expression of several undefined antigens by western blot (Marsh et al. 1998, 1999). The monoclonal antibody 6C11 produced against *N. caninum* protein SAG1 does not react with *N. hughesi* SAG1 (Marsh et al. 1999). This is the most rapid and clear means of distinguishing the two species described to date. More diagnostic tests are needed to differentiate between *N. hughesi* and *N. caninum* in live animals in order to better understand the distribution of these two parasites.

Dense granule organelles are present in all apicomplexan parasites (Cesbron-Delauw 1994). After the parasite has penetrated the host cell and established the parasitophorous vacuole (PV) the protein contents of the dense granule organelles, the GRA proteins, are released. These proteins are specifically targeted to the PV membrane, or the vacuolar space. Dense granule proteins targeted to the vacuole membrane participate in nutrient uptake and waste excretion exchanges between the parasite and the host cell (Cesbron-Denlauw 1994). The dense granule proteins are antigenic and are detected by serum antibodies of infected animals. The host is exposed to dense granule proteins when they are excreted from infected cells or when infected cells rupture as a result of overwhelming parasite infection (Fisher et al. 1998). Recombinant forms of the dense granule proteins GRA6 and GRA7 are the antigens used in an ELISA test for *N. caninum* infection (Lally et al. 1998). The rELISA shows no cross reactivity with sera from cattle infected with oocysts of *T. gondii* or sporocysts of *S. cruzi* (Lally et al. 1998). Difference between the dense granule proteins of the two species of *Neospora* may serve as the basis for a differential diagnostic assay that would distinguish between them.

Antibodies against dense granule proteins have also been examined for their protective value. Mice immunized with purified GRA2 or GRA5 of *T. gondii* are protected from lethal toxoplasmosis (Cesbron-Delauw 1994). Mice immunized with GRA7 of *N. caninum* are protected against lethal neosporosis (Lindsay personal communication 1999). The homology between the dense granule proteins of the two *Neospora* species needs to be evaluated to determine the potential of a simple dense granule based vaccine against both pathogens.

Materials and Methods

In vitro culture of parasites

Tachyzoites of the Nh-A1 strain of *N. hughesi* (Cheadle et al. 1999) and *Neospora caninum* NC-1, NC-2, Liverpool and Swiss strains were grown and maintained in African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Rockville, Maryland, USA). The CV-1 cells were grown to confluence in 75 cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 25 mM HEPES buffer, 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell cultures were maintained in growth medium in which the FBS content was lowered to 2%. All cell cultures were incubated at 37°C.

For general maintenance of tachyzoites, monolayers were examined with an inverted microscope for the development of lesions (areas devoid of host cells caused by parasite replication) in the monolayer or the presence of many extracellular tachyzoites. Once lesions were observed, or many extracellular parasites were present, the monolayer was scraped with a cell scraper and 5 ml of the tachyzoite containing fluid was transferred to 2 flasks of CV-1 cells. Tachyzoites were passaged in this manner every 3 to 7 days.

For use in this study, tachyzoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and magnesium. The CV-1 cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10-ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 μm filter to remove cellular debris. The numbers of tachyzoites in the filtrate was determined using a hemacytometer.

Polymerase chain reaction

To prepare parasites for use in PCR reactions, the organisms were washed in PBS and suspended in water at a concentration of 20 x 10^6 in 500μl of water. This stock was frozen at –20°C until used.

In a sterile 1.5 ml tube, 20 μl of thawed tachyzoite stock described above was added to 200 μl of Instagene matrix (BioRad) heated at 56°C for 30 minutes in a heating block, and then placed in a boiling water bath for 8 minutes. The tube was then vortexed and centrifuged for 2 minutes at high speed. From this preparation, 10 μl of the supernatant was added to a Ready-To-Go PCR bead (BioRad), 13 μl sterile water and 2.0 μl primers at 0.5 μM each primer. The mixture was topped with 50 μl of sterile mineral oil and loaded into a thermocycler (Hybaid). The thermocycler parameters consisted of 94°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for one minute, annealing for one minute at 60°C for GRA6 and 50°C for GRA7, and a one minute extension at 72°C and a final 10 minute prolonged extension at 72°C after the 40 cycles were completed.

Specific primers based on the published sequences of the NcGRA6 (AF029350) and NcGRA7 (AW119252) genes were used to amplify these genes from the tachyzoite genomic DNA (Lally et al 1997, Liddell et al 1998). Primers used to amplify GRA6 were forward primer, GRA6F and reverse primer, GRA6R. Primers specific for GRA7 were forward primer, GRA7F and reverse primer, GRA7R. Refer to table 1 for primer sequences. The PCR products were purified using the Quiagen PCR clean up kit (Quiagen).

Primer name	Primer Sequence
GRA7F	5'-GGC TTT TCC ATA TTA TTT TTC GTC GTC TCT-3'
GRA7R	5'-GAG CTC CGG AAC TCA TCG GCC AGG TAG G-3'
GRA6F	5'-GAC CGC GAG GGC TAA ACA GTG AGA AGG-3'
GRA6FI	5'-TCT TCC GAG TTA GCA GGC AGT C-3'
GRA6RI	5'-CGC GTT CCT CCG TAT CAA TCG TCA C-3'
GRA6R	5'-CCG CGG CTC TCC CAC AAG TGC ATT CTC CA -3'

Table 4.1*:* Primer sequences

Sequencing

Sequencing was carried out in the Virginia Tech DNA sequencing facility using an automated fluorescent dye sequencer (ABI systems). Internal primers were designed for GRA6 to enhance sequencing reliability of the insert. These two primer sequences, GRA6FI and GRA6RI, are shown in table 1. The sequences were confirmed by sequencing both forward and reverse complementary strands. The GRA6 gene of each *N. caninum* strain and *N. hughesi* was sequenced four separate times to confirm the high degree of variation seen in that gene. The GRA7 gene of each *N. caninum* strain was only sequenced once. The GRA7 gene of *N. hughesi* was sequenced twice.

Sequences were analyzed using the Seqman, Megaline, and Protean programs of LaserGene software package, (DNAStar Inc.). All sequence distances reported were generated in Megaline using the Clustal method of alignment and the sequence distance function.

SDS Protein Separation and Western Blot using GRA6 and GRA7 protein specific antibodies

Tachyzoites were harvested as previously described after a minimum of 2 hours in serum free medium to avoid reaction with *N. caninum* antibodies common in all bovine serum. Concentration of tachyzoites was adjusted so that one well contained $3x10^6$ tachyzoites. Samples were mixed 1:1 v/v with 95% Laemmli buffer containing 5.0% 2-β mercaptoethanol, and placed in a boiling water bath for 5 minutes. This solution was electophoresed in a 12.0% polyacrylimide SDS page gel at 25 mAmp per gel, using the BioRad Mini Protean Cell and the BioRad powerpac 300 power source, until the dye front reached the bottom of the gel. The

BioRad MultiMark™ molecular weight standard run in a separate lane as a reference for protein molecular weight determination.

Separated proteins were transferred to a 0.22 μm pore nitrocellulose membrane using the BioRad transblot SD semi dry transfer cell at 70mAmps for 45 minutes. The blotted membrane was blocked for 18 hours at room temperature in 2.0% bovine serum albumin (BSA) in TBS (pH 7.4) and immediately placed in specific rabbit sera raised against either NcGRA6 or NcGRA7 at a dilution of 1:200 in TBS and placed on a shaker for 4 hours at room temperature. The membrane was washed 2 times for 5 minutes each in TBS on an orbital-shaking platform. The vectastain anti-rabbit ABC kit was used to detect rabbit antibodies (Vectastain). The immunoblot was washed as previously described and developed using a mixture of 4-chloro-1-napthol, TBS, MeOH and H_2O_2.

Results

Sequence analysis

The nucleotide sequence for NhGRA6 showed a consistent 4.0% difference from the *N. caninum* strains examined, variation among the *N. caninum* strains themselves was only 0.0-0.3%. There were 24 nucleotides approximately 540 bases into the coding region of the NhGRA6 gene that did not correspond to the NcGRA6 genes (figure 1 follows text). Sequences aligned again after this region and the primer binding site, which was located after the stop codon in both the NcGRA6 and NhGRA6 genes remained intact. The band migration of the PCR product for NhGRA6 was approximately 850 bp, which was visibly slower than the NcGRA6 products, which migrated to approximately 800 bp (figure 2). The deduced amino acid sequence for NhGRA6 was 8.8-9.4% different from the NcGRA6 deduced protein of all *N. caninum* strains in the regions that could be compared, and was 33 amino acids longer than any of the *N. caninum* proteins (figure 3 follows text). Variation among the NcGRA6 amino acid sequences was 0.0-0.5%. The two described hydrophobic regions at residues 17-32 and 155-172 in NcGRA6 (Liddell et al. 1998) are conserved in NhGRA6.

The nucleotide sequence for NhGRA7 showed a consistent 6.0% difference from all strains of *N. caninum* examined, variation among the *N. caninum* strains was 0.2%. The deduced amino acid sequence for NhGRA7 showed a consistent 14.8% difference from *N. caninum* strains, where as the variation among the *N. caninum* strains was only 0.5-0.9% (figure 4 follows text). All deduced amino acid sequences for GRA7 were approximately 200 amino acids long. The three hydrophobic regions at residues 7-28, 98-111 and 139-160 are conserved (Lally et al. 1997).

Western blot

The major protein recognized by polyclonal monospecific antibodies raised against GRA6 in the *N. caninum* preparation was approximately 37kDa as has been reported previously (Liddell et al. 1998), and the corresponding band in the *N. hughesi* preparation showed an approximate MW of 42 kDa (figure 5). A smaller protein was weakly recognized by the

antibodies in each preparation, of approximately 31 kDa in the *N. caninum* preparation and 37 kDa in the *N. hughesi* preparation.

Western blot using polyclonal monospecific antibodies raised against recombinant NcGRA7 produced similar immunoblot results for *N. hughesi* and *N. caninum* . The major protein recognized by these antibodies was approximately 33kDa, which agrees with previous reports. A slight difference in *N. hughesi* band migration was observed (figure 6). Two additional bands of weights less than 33kDa were weakly recognized in both the *N. hughesi* and *N. caninum* tachyzoite antigen lanes that have not been described in previous reports.

Discussion

The NcGRA6 and NcGRA7, are antigenic proteins unique to *N. caninum* which were isolated by screening an *E. coli* cDNA library of *N. caninum* genes (Lally et al. 1996, 1997,Hemphil et al. 1998, Liddell et al. 1998). Sequencing of the genes encoding these proteins has been completed (Genebank access numbers AW119252, and AF029350).

The genes of two conserved antigens of the dense granule family of proteins were sequenced and compared between *N. hughesi* and *N. caninum.* The GRA6 and GRA7 proteins of *N. caninum* are homologous to those of *T. gondii*. They have conserved signal sequences and hydrophobic domains indicating that their functions may be similar within the two groups of parasites. The GRA6 and GRA7 of *T. gondii* and *N. caninum,* share 32.0 % and 28.4% similarity respectively as determined by the Clustal method of alignment in Megaline. *Neospora hughesi* GRA6 and GRA7 share 30.4% and 28.9% similarity with the homologous proteins of *T. gondii* as shown by the Clustal method in Megaline. The variation between *T. gondii* and each of the *Neospora* species appears to be similar.

In *T. gondii* infected cells, TgGRA7 is associated with the PV membrane and can be detected extending into the host cell cytoplasm (Jacobs et al. 1997). TgGRA7 has also been detected in the supernatant of infected cells and is thought to be an indicator of intracellular infection with *T. gondii* (Fischer et al. 1998). NcGRA7 is thought to play a role in penetration of the host cell and maintenance of the parasitophorous vacuole membrane and is expressed by both the tachyzoite and bradyzoite stages of *N. caninum* (Hemphill et al. 1998).

Both the *T. gondii* and the *N. caninum* GRA6 have a hydrophobic region that is likely a transmembrane domain suggesting that the protein is associated with and may span the PV network membranes (Lecordier et al. 1995, Liddell et al. 1998).

This degree of variation between *N. caninum* and *N. hughesi* reported here is consistent with variation observed in other genes between the two species. The 14.8% variation in the GRA7 protein is the greatest degree of variation described so far between proteins of the two species so far. There is an additional 33 amino acid segment at the carboxyl end of the NhGRA6 deduced protein that could not be considered in the alignment comparison and is not represented in the reported 9.0% variation. The size variation in the genes for *N. caninum* and *N. hughesi* GRA6 was visually demonstrated by a slower migration of the NhGRA6 from the PCR amplification as seen in a 1.0% agarose gel (figure 2). The size variation in the GRA6 proteins

of *N. caninum* and *N. hughesi* was confirmed by 10.0% SDS PAGE gel and western blot analysis using antibodies specific for NcGRA6. The differences in the GRA6 and GRA7 genes and deduced proteins of *N. hughesi* and *N. caninum* provide additional support to the distinction of *N. hughesi* as a separate species. The visible difference in migration of the GRA6 proteins in SDS gel could serve as a diagnostic tool in differentiating between the two species.

The additional 33 amino acid segment present in the NhGRA6 protein has strong antigenic potential in three areas according to the Jameson-Wolf index (Protean). It is highly hydrophilic according to the Kyte-Doolitle index (Protean) and highly flexible due to a 30% content of glycine residues in this section. This additional segment may provide the basis for the development of a monoclonal antibody capable of distinguishing between the two *Neospora* species.

The primers used are specific to *Neospora* species and did not produce product when *T. gondii or S. neurona* DNA was added as template. This assay may eventually be used on CSF in cases of EPM where *Neospora* species are suspected. The nucleotide sequence data and demonstrated difference between the sizes of the GRA6 genes and proteins of *N. hughesi* and *N. caninum* provide additional methods of distinguishing between the two species of *Neospora* that will aid future research and the development of diagnostic tests.

Acknowledgements: Lee Weigt at the Virginia Tech DNA sequencing facility for sequencing work.

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Figure 4.1: Alignment of nucleotides of GRA6 from *N. caninum* strains and *N. hughesi*. Alignment starts at 630 bp and illustrates inserts into the NhGRA6 gene. In frame stop codons are boxed with dark lines. Nucleotides in the NhGRA6 that differ from *N. caninum* strains are boxed with light lines.

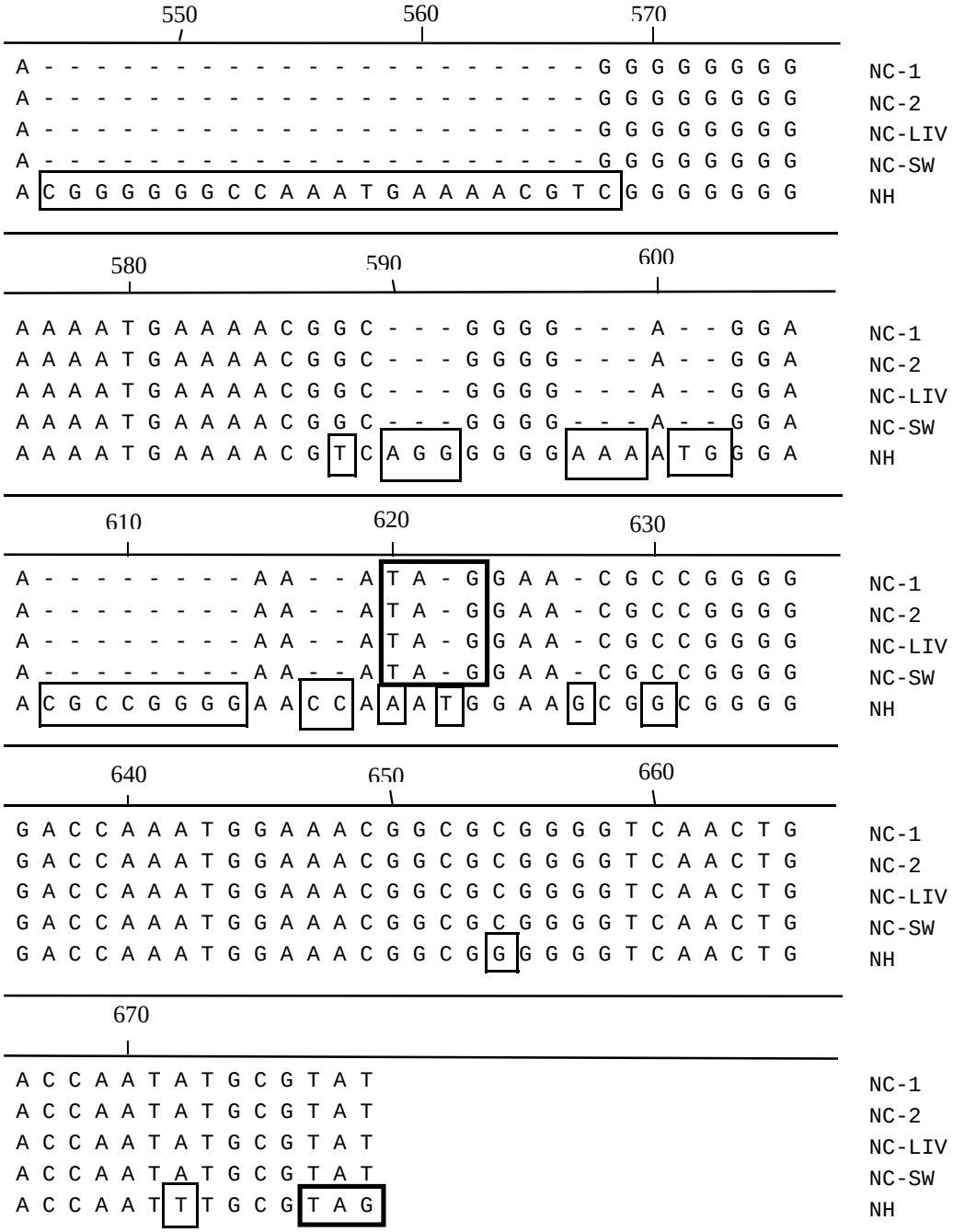


Figure 4.2: PCR products of amplification with GRA6 specific primers; *N. hughesi* migrates to approximately the 850 bp position while *N. caninum* migrates to approximately the 800 bp position.

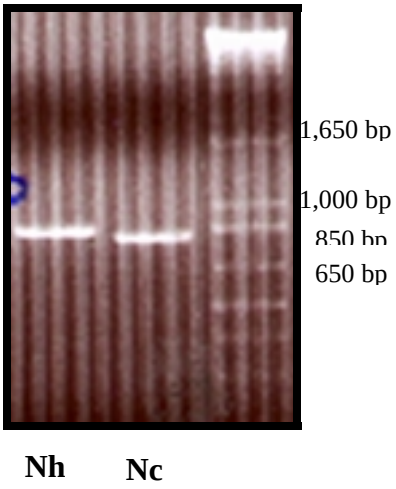


Figure 4.3: Deduced amino acid sequences for GRA6 from *N. caninum* strains and *N. hughesi.*

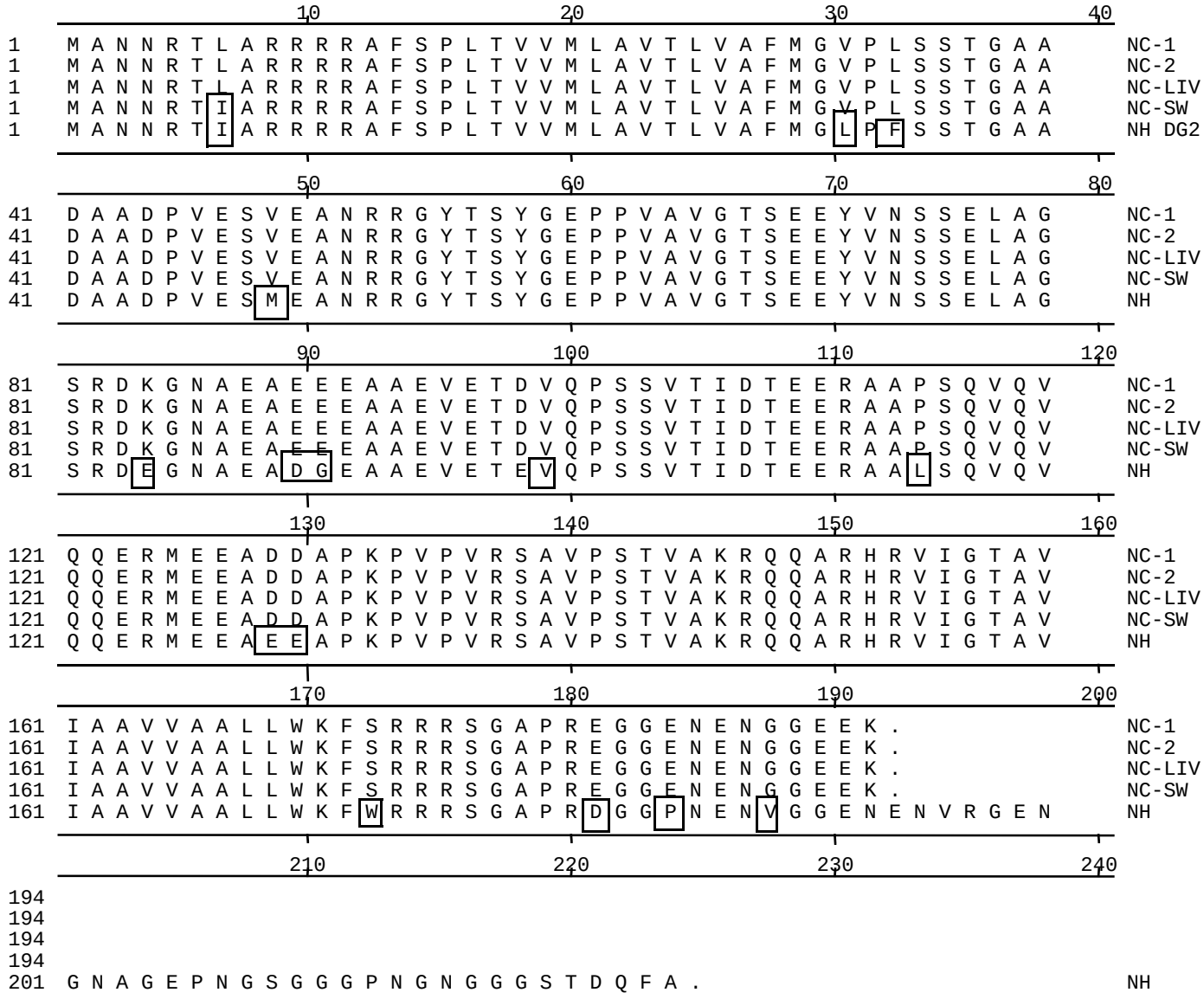


Figure 4.4: Deduced amino acid sequences for GRA7 from *N. caninum* strains and *N. hughesi.*

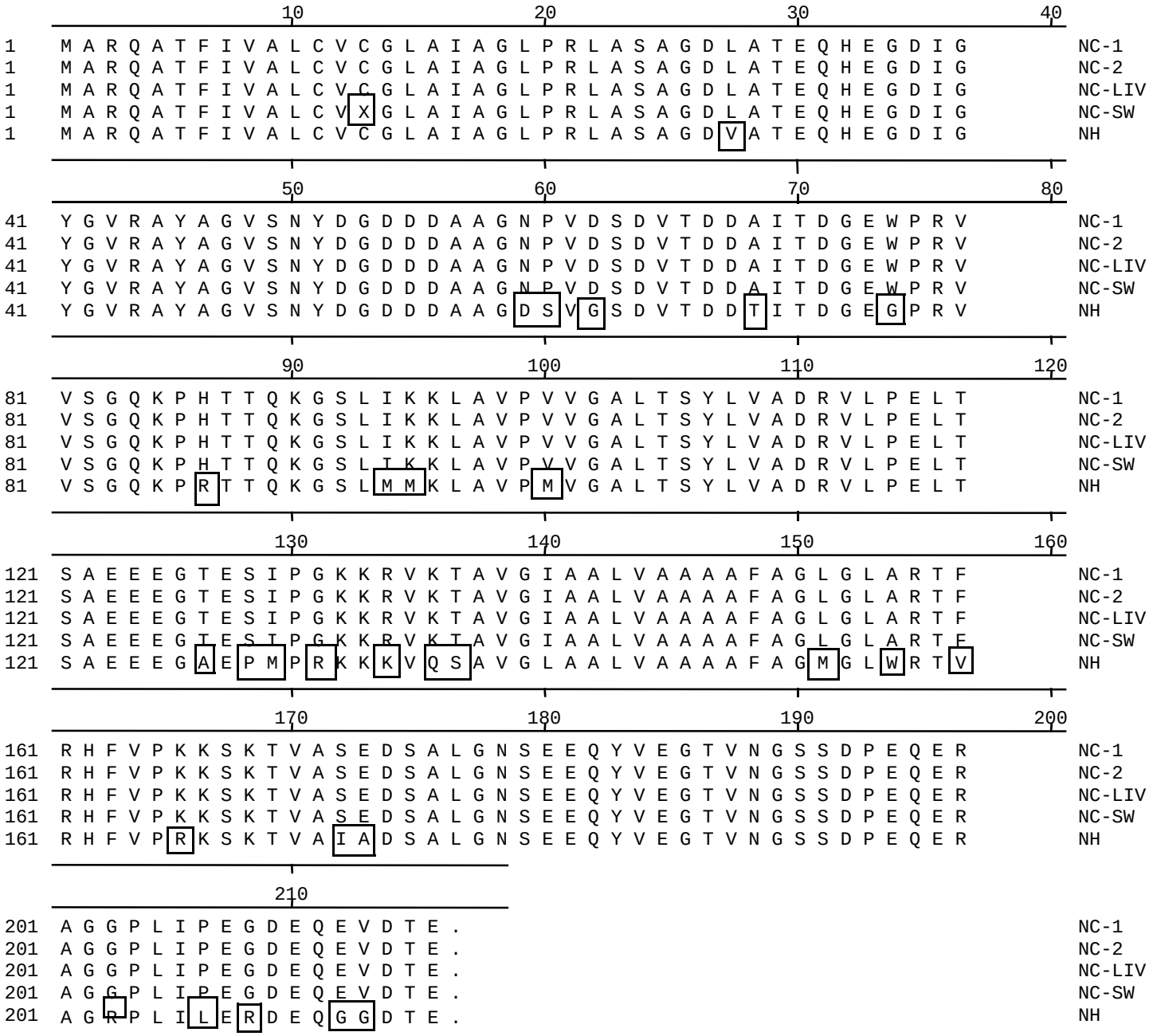


Figure 4.5: Western Blot using GRA6 monospecific antibodies. Lane one contains the molecular weight standard marker. Lane 2 contains whole *N. hughesi* tachyzoite preparation. Lane 3 contains *N. caninum* whole tachyzoite preparation.

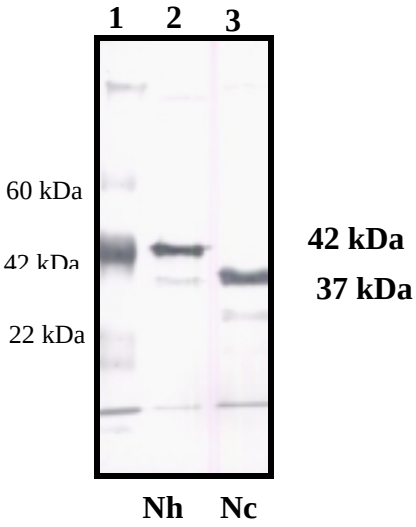
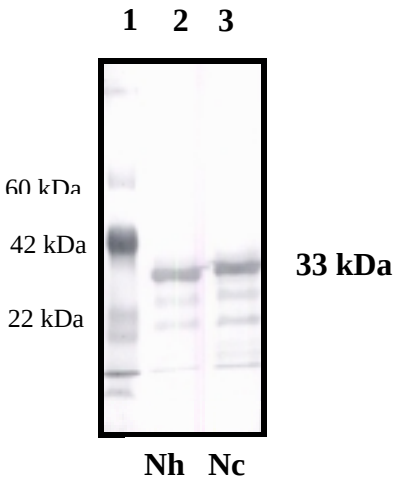


Figure 4.6: Western Blot using GRA7 monospecific antibodies. Lane one contains the molecular weight standard marker. Lane two contains *N. hughesi* whole tachyzoite preparation. Lane 3 contains *N. caninum* whole tachyzoite preparation.



Chapter 5: Summary

The research presented in this thesis supports the identification of *Neospora hughesi* as a separate species from *Neospora caninum* and provides novel methods for distinguishing the two species. The original distinction was made in 1998 by Marsh et al. and was based on morphologic, antigenic and genetic characteristics. This thesis contains the first report of differential activity of the two species of *Neospora* in rodent models and in dogs as well as the first description of the GRA6 and GRA7 genes and proteins of *N. hughesi*.

The present study provides information on the susceptibility of strains of mice and gerbils to *N. hughesi* neosporosis. It demonstrates that, of the rodent species and strains investigated, only interferon gamma gene knockout BALB/c (γ-IFNKO) mice were susceptible to severe disease caused by *N. hughesi* and that the predilection site was markedly different from that of *N. caninum*. In γ-IFNKO mice *N. hughesi* exhibits a unique tropism to the heart which leads to a distinctive pathology marked by necrotizing myocarditis with atrial thrombosis and fibroplastic replacement of myocytes, as well as lesions and inflammation in other organs and which eventually leads to the death of the mice. In γ-INFKO mice *N. caninum* causes multifocal coalescing hepatic and splenic necrosis, pneumonia and rapid death. This report supports previous research indicating that interferon gamma is an essential mediator of resistance to infection with *Neospora* species (Long et al. 1998, Baszler et al. 1999). The drastic difference in site of predilection between the 2 *Neospora* species provides further validation of the distinction between them. The specific predilection site of *N. hughesi* in γ-IFNKO mice separates this infection from those caused by closely related organisms that could be confused while using other assays. We propose that because of its unique, obvious and consistent pathogenicity, the γ-IFNKO mouse model be used as a bioindicator of *N. hughesi* infection. There is sufficient time between the onset of disease and death for intervention as well as obvious clinical signs, making this model valuable for the preliminary testing of therapies to be used in horses with *N. hughesi* EPM.

Infections in BALB/c, CD-1, C57BL/6 mice and gerbils with *N. hughesi* are less severe than infections with *N. caninum*. In these rodents, infections with *N. caninum* produce signs of central nervous disease and mortality. The BALB/c, immunosuppressed CD-1 (or outbred mice) and gerbils are used as standard models of *N. caninum* CNS disease. In contrast, no clinical signs or mortality were observed in any of these rodents following *N. hughesi* infection. Infections with *N. hughesi* in these rodents produced no lesions or minimal lesions and no tissue specificity was seen in the various mouse strains or gerbils. The difference in pathogenicity in these types of rodents provides an additional biological distinction between the two species of *Neospora*.

Dogs fed the brains of *N. hughesi* infected CD-1 mice did not shed *N. hughesi* oocysts detectable by fecal flotation and γ-INFKO mice fed concentrated fecal material from these dogs did not die or develop antibody titers to *Neospora* species. Presence and activity of parasites in the innoculum was confirmed by recovery of *N. hughesi* organisms from cell cultures and γ-IFNKO mice infected with the homogenized brains of infected CD-1 mice. This suggests that dogs may not be the definitive host of *N. hughesi*. However, not all dogs excrete *N. caninum* oocysts after being fed tissue cysts (McAllister et al. 1998). Additional studies in dogs need to be conducted to determine their role in the transmission of *N. hughesi*. It is interesting to note that equine neosporosis has only been observed in the Americas (Dubey and Porterfield 1990,

Daft et al. 1997, Gray et al. 1996, Lindsay et al. 1996, Marsh et al. 1996, Hamir et al. 1998). This may indicate that the definitive host of *N. hughesi* is limited to the Americas.

The genes of two conserved antigens of the dense granule family of proteins were sequenced and compared between *N. hughesi* and *N. caninum.* These proteins have conserved signal sequences and hydrophobic domains indicating that their functions may be similar within the two groups of parasites. After apicomplexan parasites penetrate the host cell and establish the parasitophorous vacuole (PV), the protein contents of the dense granule organelles (the GRA proteins) are released. These proteins are specifically targeted to the PV membrane, or the vacuolar space. Dense granule proteins targeted to the vacuole membrane participate in nutrient uptake and waste excretion exchanges between the parasite and the host cell (Cesbron-Denlauw 1994). The dense granule proteins are antigenic and are detected by serum antibodies of infected animals. The host is exposed to dense granule proteins when they are excreted from infected cells or when infected cells rupture as a result of overwhelming parasite infection (Fisher et al. 1998). Recombinant forms of the dense granule proteins GRA6 and GRA7 are the antigens used in an ELISA test for *N. caninum* infection (Lally et al. 1998). Difference between the dense granule proteins of the two species of *Neospora* may serve as the basis for a differential diagnostic assay that would distinguish between them.

Differences were observed in the GRA6 and GRA7 proteins of the two *Neospora* species by amplification and sequencing of the genes. A 4.0% difference in nucleotide sequence and a 9.0% difference in putative amino acid sequence were demonstrated in GRA6. The variation in the GRA6 deduced protein for the N. caninum strains examined was 0-0.5%. A 6.0% difference in nucleotide sequence and a 14.8% difference in deduced amino acid composition were demonstrated in GRA7. The variation in the GRA7 deduced amino acid sequences for the *N. caninum* strains examined was 0.5-0.9%. The dense granules GRA6 and GRA7 are homologous antigens of *T. gondii* and *N. caninum*, which share 32.0 % and 28.4% similarity respectively when analyzed using the Clustal method of alignment. *Neospora hughesi* GRA6 and GRA7 share 30.4% and 28.9% similarity with the homologous proteins of *T. gondii* in the Clustal method. The variation between *T. gondii* and each of the *Neospora* species appears to be similar.

This degree of variation between *N. caninum* and *N. hughesi* is consistent with variation observed in other genes between the two species. The variation in the GRA7 protein is the greatest degree of variation observed so far between proteins of the two species. There is an additional 33 amino acid segment at the carboxyl end of the NhGRA6 deduced protein that could not be considered in the alignment comparison and is not represented in the reported 9% variation. Variation in the genes for *N. caninum* and *N. hughesi* GRA6 were visually demonstrated by different migrations of the PCR amplicons in a 1% agarose gel. Variation in the GRA6 proteins of *N. caninum* and *N. hughesi* were confirmed by SDS page 10% acrylimide gel and western blot analysis using antibodies specific for NcGRA6. A 6.0% difference in nucleotide sequence and a 14% difference in putative amino acid sequence were demonstrated in GRA7. The differences in the GRA6 and GRA7 genes and deduced proteins of *N. hughesi* and *N. caninum* provide additional support to the distinction of *N. hughesi* as a separate species. The visible difference in migration of the GRA6 proteins in SDS gel can serve as a diagnostic tool in differentiating between the two species.

The additional 33 amino acid segment present in the NhGRA6 protein has strong antigenic potential in three areas according to the Jameson-Wolf index (Protean). It is highly hydrophilic according to the Kyte-Doolitle index and highly flexible due to a 30% content of glycine residues. This additional segment may be the basis of a monoclonal antibody diagnostic capable of distinguishing between the *Neospora* species.

The primers used are specific to *Neospora* species and did not produce product when *T. gondii or S. neurona* DNA was added as template. This assay may eventually be used to test CSF from cases of EPM where *Neospora* species are suspected. The nucleotide sequence data and demonstrated differences between the sizes of the GRA6 genes and proteins of *N. hughesi* and *N. caninum* provide additional methods of distinguishing between the two species of *Neospora* that will aid future research and diagnoses.

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PRESENTATIONS:
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of Veterinary Medicine Tenth Annual research Symposium. June 3

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Walsh, C.P., A.M. Zajac, C.T. Larsen, and D.S. Lindsay. 1999. *Cochlosoma anatis*: Ultrastructure and attachment. 71st Northeastern Conference on Avian Diseases. (Blacksburg, VA, June 18)

Walsh, C.P., A. M. Zajac, C.T. Larsen, and D.S.Lindsay. 1999. Attempted in vitro culture and in vivo treatment of *Cochlosoma anatis*. American Association of Veterinary Parasitologists Annual Meeting. (New Orleans, LA, July 12)

Lindsay, D.S., C.P. Walsh, S. A. Ahmed, C. C. Dykstra, A. Nichols, and J. P. Dubey. 2000. *Sarcocystis neurona* infections in mice with deleted nitric oxide synthase genes Southeastern Society of Parasitologists joint meeting with the Association of Southeastern Biologists . (Chattanooga, TN, April 5-8).

Walsh, C.P., D.S. Lindsay, A.M. Zajac, R.B. Duncan, Jr., and B.L. Blagburn. 2000. Experimental *Neospora hughesi* infections in mice. Southeastern Society of Parasitologists joint meeting with the Association of Southeastern Biologists (Chattanooga, TN, April 5-8).

PUBLICATIONS:

Walsh, C. P., S. E. Hammond, A. M. Zajac, and D. S. Lindsay. 1999. Survival of *Toxoplasma gondii* tachyzoites in goat milk. J. Eukaryotic Micro. 46: 73S-74S.

Walsh, C.P., R. B. Duncan, A. M. Zajac, B. L. Blagburn and D. S. Lindsay. 2000. *Neospora hughesi:* Experimental infections in mice, gerbils, and dogs. Vet. Parasitol. (in press).