SELWYN ARLINGTON HEADLEY

PATOLOGIA E CARACTERIZAÇÃO MOLECULAR DE *NEORICKETTSIA HELMINTHOECA* EM CÃES NO BRASIL

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Tese apresentada ao Programa de Pós-Graduação em Ciência Animal (área de concentração em Sanidade animal), da Universidade Estadual de Londrina como requisito parcial para obtenção do título de Doutor em Ciência Animal.

Orientador: Prof. Dr. Odilon Vidotto
Co-orientador: Prof. Dr. J. Stephen Dumler

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Aos meus queridos pais, mesmo que não estejam
comigo, a minha imensa gratidão.
Alguém só pode entrar na floresta até a metade,
dali pra frente está somente saindo.
(Autor desconhecido).

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BIOGRAFIA DO AUTOR

Selwyn Arlington Headley nasceu na cidade de Georgetown, Guiana. Cresceu e completou sua educação, até o nível técnico, na Guiana, onde obteve o Diploma em Agricultura pela Guyana School of Agriculture em 1984.

Ingressou no Curso de Medicina Veterinária, Universidade Federal de Santa Maria (UFSM) em 1991, e graduou-se Médico Veterinário em 1996. É Patologista Animal, tendo concluído o Curso de Mestrado em Medicina Veterinária (área de concentração de Patologia Animal) pela UFSM em 1999, sob a orientação da Professora Dominguita Lühers Graça.

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Entre 2003 a 2005 foi docente no Integrado (Faculdades de Campo Mourão), onde ministrou as disciplinas de Anatomia Patológica Geral, Anatomia Patológica Especial e Histologia Veterinária, e era responsável pelas atividades realizadas no Laboratório de Patologia Animal.

A atual linha de pesquisa do autor está direcionada à epidemiologia e à patogenia das infecções induzidas por organismos do gênero *Neorickettsia* em mamíferos. O autor tem diversos artigos publicados em revistas nacionais e internacionais de impacto sobre vários aspectos de patologia e saúde pública.

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RESUMO

Neorickettsia helminthoeca, uma bactéria intracelular obrigatória, é a causa da doença conhecida como intoxicação por salmão (*salmon poisoning disease* - SPD) dos canídeos. O trematódeo digenético, *Nanophyetus salmincola*, é o vetor desta bactéria, no qual o patógeno é mantido no seu ciclo biológico, que inclui um caramujo (*Oxytrema silicula*), peixes (principalmente, salmão e a truta), e canídeos (cães e outros mamíferos que se alimentam de peixes). O *O. silicula* é endêmico somente em alguns rios no Pacífico Nordeste dos Estados Unidos, resultando na ocorrência restrita na SPD. Esse trabalho descreve as alterações patológicas e os achados da biologia molecular associados à *N. helminthoeca* em cães do Brasil. Entre 2001 a 2002 vinte cães adultos (5 cães sem raça; 15 Beagles), de ambos os sexos, da cidade de Maringá, Paraná, apresentaram alterações macroscópicas e histológicas consistentes com aquelas descritas na SPD. As alterações intestinais foram caracterizadas por hiperplasia intensa do tecido linfóide, hemorragia e congestão com a presença de organismos consistentes com *Neorickettsia* spp. nas células reticuloendoteliais. Esses organismos foram mais facilmente visualizados na coloração histológica de Giemsa. As alterações macroscópicas foram caracterizadas por linafadenopatia generalizada, principalmente nos linfonodos mesentéricos, pré-escapulares e poplíteos, com vários folículos esbranqüiçados aleatoriamente distribuídos na região cortical; hipertrofia intensa das placas de Peyer e do tecido linfóide intestinal; hemorragia intestinal e espenomegalia. A avaliação microscópica das lesões dos linfonodos revelou edema e depleção cortical intenso e proliferação das células reticuloendoteliais associado aos organismos intracitoplasmáticos consistentes com a *Neorickettsia* spp nas células reticuloendoteliais. As alterações intestinais foram caracterizadas por hiperplasia do tecido linfóide, hemorragia e congestão com organismos intralesionais intracitoplasmáticos consistentes com a *Neorickettsia* spp nas células reticuloendoteliais. Esses organismos foram facilmente observados na coloração de Giemsa. Tecidos selecionados (linfonodos, intestino, baço e fígado) de 10 cães foram coletados assepticamente na necropsia e processados para avaliação por PCR, clonagem, seqüenciamento e análise filogenética. A *N. helminthoeca* foi confirmada na PCR com os primers específicos para 16S ribossomal RNA (*rrs*) gene do gênero *Neorickettsia* em dois cães (Maringá 1, linfonodo mesentérico; Maringá 2, intestino, placa de Peyer); esses cães também foram positivos na PCR para os genes da β-subunidade da RNA polimerase (*rpo*B) e da proteína de choque-térmico (*gro*ESL). As seqüências de nucleotídeos do DNA do cão Maringá 1 apresentaram identidade de 100% quando foram comparadas com aquelas da *N. helminthoeca* depositadas no GenBank para os *rrs*, *rpo*B e *gro*ESL genes. Essa é a primeira descrição da *N. helminthoeca* e da intoxicação por salmão fora da região endêmica dos Estados localizados ao Nordeste do Pacífico dos EUA e do Canadá. A forma de transmissão e de infecção e o ciclo biológico associados a *N. helminthoeca* no Brasil são atualmente desconhecidos.

Palavras-chave: Cães. *Neorickettsia helminthoeca.* Intoxicação por salmão. Patologia. Biologia molecular.

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ABSTRACT

Neorickettsia helminthoeca, an intracellular obligate bacterium, is the cause of salmon poisoning disease (SPD) of canids. The digenean trematode, *Nanophyetus salmincola*, is the vector of the bacterium, which maintains the pathogen throughout its entire life cycle, that includes a river snail (*Oxytrema silicula*), fishes (principally, salmon and trout), and canids (dogs and other fish-eating mammals). The snail, *O. silicula,* is endemic only in certain rivers of the Pacific Northwest of the USA which has resulted in the restricted occurrence of SPD. This study describes the pathological alterations and the molecular findings associated with *N. helminthoeca* in Brazilian dogs. During 2001 to 2005, 20 adult dogs (5, mongrels; 15 Beagles), of both sexes, from the city of Maringá, Paraná, demonstrated gross and histological alterations consistent with those described in SPD. Gross lesions were characterized by generalized lymphadenopathy, predominantly at the mesenteric, pre-scapular, and popliteal lymph nodes, with several white follicles randomly distributed at the cortical zone; severe hypertrophy of intestinal Peyer's patches and intestinal lymphoid tissue; intestinal hemorrhage, and splenomegaly. Microscopic evaluation of lymph nodes lesions revealed severe cortical edema and depletion, and proliferation of reticuloendothelial cells associated with intracytoplasmic neorickettsial organisms within reticuloendothelial cells. Intestinal lesions were characterized by severe hyperplasia of lymphoid tissue, hemorrhage, and congestion with intralesional intracytoplasmic neorickettsial organisms in reticuloendothelial cells. These organisms were readily demonstrable by Giemsa staining. Selected tissues (lymph node, intestine, spleen, and liver) from 10 Beagles were harvested aseptically and processed for PCR evaluation, cloning, sequencing, and phylogenetic analyses. *N. helminthoeca* was confirmed by PCR using *Neorickettsia* genus-specific 16S ribosomal RNA (*rrs*) primers in two dogs (Maringá 1, mesenteric lymph node; Maringá 2, Peyer's path); these dogs were also positive by PCR for the RNA polymerase *β*-subunit (*rpo*B*)* and the heat shock protein (*gro*ESL) genes of *N. helminthoeca*. The DNA nucleotide sequences of the *N. helminthoeca* Maringá 1 dog were 100% identical to that of the *N. helminthoeca* sequences deposited in GenBank for the *rrs*, *rpo*B, and *gro*ESL genes. This is the first description of *N. helminthoeca* and SPD beyond the endemic region of the Northwest Pacific States of the USA, and Canada. The method of transmission-infection and the biological life cycle associated with *N. helminthoeca* in Brazil are currently unknown.

Keywords: Dogs. *Neorickettsia helminthoeca.* Salmon poisoning disease. Pathology. Molecular biology.

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1 LITERATURE REVIEW

1.1 INTRODUCTION

Salmon poisoning disease (SPD) is an acute, febrile, and fatal disease of canids that is caused by *Neorickettsia helminthoeca* (GORHAM & FOREYT, 1998; DUMLER et al 2005). The disease is endemic to certain specific geographical regions of the Northwest Pacific of the United States of America and Canada (SIMMS et al., 1931; BOOTH et al., 1984; GORHAM & FOREYT, 1998).

The first written description of SPD was registered in "Henry's Astoria Journal" and occurred in the Northwest region of Oregon, USA in 1814 (MILLEMANN & KNAPP, 1970). In 1849 Thornton (MILLEMANN & KNAPP, 1970) related that uncooked salmon given to dogs resulted in sudden death. Cooper and Suckley in 1859 (MILLEMANN & KNAPP, 1970) described that 90% of the dogs died suddenly after eating fresh salmon and that surviving dogs were immune to the disease, but claimed that these dogs were infected by canine distemper.

In 1911 Emile F. Pernot thought that SPD was induced by an amoeba and described the disease for the first time after experimental reproduction of clinical manifestations and gross lesions of the disease in dogs infected by the administration of contaminated fish (SIMMS et al., 1931; PHILIP, 1955; MILLEMANN & KNAPP, 1970).

Thereafter, the etiological agent was attributed to a bacterium (Bonebrake, 1925 *apud* BENNINGTON & PRATT, 1960), or associated with mechanical destruction (Hoeppli 1926 *apud* BENNINGTON & PRATT, 1960). Trematodes observed in the intestine of dogs with SPD made DONHAM (1924) doubt the previously mentioned hypotheses, and suggested that cysts found in fishes and dogs might be related to the same agent and the cause of SPD (DONHAM, 1925).

The intestinal trematode was then considered responsible for SPD (DONHAM et al., 1926), and was described and named *Nanophyetus salmincola* (CHAPIN, 1926). Experimental studies demonstrated that the dog, coyote, and fox are highly susceptible to SPD (DONHAM & SIMMS, 1927). A river snail, identified as *Goniobasis plicifera* var *silicula* Gould, that was only observed in the streams within the endemic regions of SPD was considered as the principal factor that maintained SPD within that specific geographical area

(SIMMS et al., 1931). The snail was later re-classified as *Oxytrema silicula* (WALLACE, 1935).

CORDY & GORHAM (1950) described the clinical manifestations and pathological alterations of SPD in dogs and foxes experimentally infected by the ingestion of contaminated salmon and by intraperitoneal inoculation made from suspensions of lymphoid and splenic tissue from previously infected dogs. These authors described intracytoplasmic inclusion elementary bodies within macrophages of the spleen and lymph nodes and concluded that the agent seen was morphologically similar to a *Rickettsia*.

In 1953, at the IV International Congress of Microbiology, Rome, Italy, the intracellular organism previously described as *Rickettsia*-like by CORDY & GORHAM (1950) was considered to be significantly different from *Rickettsia* and the name *Neorickettsia helminthoeca*[1] was suggested (Philip et al 1953 *apud* PHILIP et al., 1954a); since it was a new *Rickettsia* maintained within a worm. This was the first time in the history of microbiology that an obligatory, helminth-borne pathogen was described (PHILIP et al., 1954a).

PHILIP et al., (1954b) suggested that *N. salmincola*, *O. silicula*, salmon, and canids' maintained *N. helminthoeca*, but only the latter died from the disease; this gave rise to various studies relative to the biology of *N. salmincola*. Between the years 1960 to 1970, various authors (BENNINGTON & PRATT, 1960; GEBHARDT et al., 1966; NYBERG et al., 1967; MILLEMANN & KNAPP, 1970) described aspects of the biological cycle of *N. salmincola* and their association with SPD. At around this same time, the histological and histochemical effects of *N. salmincola* on the snail were described (PORTER et al (1967).

A disease with similar biological cycle, clinical signs, and pathological manifestations of SPD, but with a larger host range (canids and bears), was observed in the Elokomin River, State of Washington, USA, and named Elokomin Fluke Fever - EFF (FARRELL et al., 1973). Studies using complement fixation and fluorescent antibodies were then made to differentiate the EFF agent from *N. helminthoeca* (SAKAWA et al., 1973; FRANK et al. 1974). Fluorescent antibody studies demonstrated that the EFF and SPD agents are different from *N. sennetsu* (KITAO et al., 1973). However, EFF is thought to be identical to SPD and the agent of EFF may possibly be a strain of *N. helminthoeca* (RIKIHISA et al. 2005). Additionally, the agent of EFF is not recognized as a distinct species in the Bergey's

[1] The word helminthoeca (Greek origin, helminthos + oikos) signify to live within a worm. In the article published in 1953 (PHILIP et al, 1954) the name used was *N. helminthẽca*, which was then changed to maintain the etymology of the word.

Manual of Systematic Bacteriology (DUMLER et al. 2005).

BROWN et al (1972) cultivated *N. helminthoeca* in macrophages, giving rise to the era of immunological and molecular studies of this organism. However, it was only in 1991 that *N. helminthoeca* was isolated, continually propagated and maintained in cell culture derived from canine macrophages, DH82 cells (RIKIHISA et al., 1991). It was then demonstrated by immunofluorescence and western blot studies that there is antigenic cross-reactivity between *N. helminthoeca* and some *Ehrlichia* species (*N. risticii* previously known as *E. risticii*; *N. sennetsu* previously known as *N. sennetsu*; and to some extent *E. canis*), concluding that these organism are closely related (RIKIHISA, 1991a). This antigenic relationship was further confirmed by phylogenetic studies using the 16S ribosomal RNA (16S rRNA; *rrs*) gene where it was demonstrated that *N. helminthoeca*, *N. risticii*, and *N. sennetsu* were more closely related than other then known *Ehrlichia* species (PRETZMAN et al., 1995). The citrate synthase gene (*glt*A) was then proposed as another comparative method for phylogenetic analysis and identification of members of the then *Ehrlichia* group (INOKUMA et al., 2001).

Prior to this, all *Ehrlichia* species, *Cowdria ruminantium*, and *Neorickettsia helminthoeca* were considered members of the *Ehrlichieae* tribe, and *Anaplasma* species along with *Aegyptianella*, *Haemobartonella*, and *Eperythrozoon* formed the Anaplasmataceae family. After the molecular characterization, "ehrlichiae" were then reorganized into three principal subgroups: I) *E. canis, E. chaffeensis, E. ewingii, E. muris*, and *Cowdria ruminantium*; II) *E. equi, E. phagocytophila, E. platys,* and *Anaplasma marginale*; and III) *E. sennetsu, E. risticii, N. helminthoeca*, and the *Stellantchasmus falcatus* (SF) Agent (WALKER and DUMLER, 1996). Based on this analysis and additional phylogenetic studies using the *rrs* and the heat shock protein (*gro*ESL) genes, a major reorganization of the Rickettsiales families *Rickettsiaceae* and *Anaplasmataceae* was realized: the tribe structure of *Rickettsiaceae* was abolished, and it was proposed that four major bacterial genera be formed (*Anaplasma, Ehrlichia, Neorickettsia*, and *Wolbachia*) within the family *Anaplasmatacaeae* (DUMLER et al., 2001). Phylogenetic studies using the RNA polymerase *β*-subunit (*rpo*B) gene (TILLARDAT-BISCH et al. 2003) and the *p51* gene (RIKIHISA et al 2004) offered other methods to confirm and analyze members of the *Anaplasmataceae* family.

HEADLEY et al (2004) observed gross and histopathological lesions in 10 dogs from the city of Maringá, Paraná, Brazil, that were consistent with those described in SPD and concluded, based on morphological characteristics, that the infectious agent was similar to *N. helminthoeca*. Two years later this hypothesis was confirmed by PCR using

Neorickettsia genus *rrs* primers and *N. helminthoeca*specific primers based targeting the *rpo*B and *gro*ESL genes; moreover, the DNA sequences from one dog were 100% similar to those of *N. helminthoeca* for the *rrs*, *rpo*B, and *gro*ESL genes (HEADLEY et al 2006). This is the first case of *N. helminthoeca* diagnosed beyond the endemic region of the Pacific Northwest of the USA.

1.2 MORPHOLOGY, TAXONOMY AND PHYLOGENETIC RELATIONSHIP

N. helminthoeca is a coccoid or pleomorphic cell that resides within cytoplasmic vacuoles of macrophages of canids (RIKIHISA et al 2005). The intracytoplasmic organism is Gram-negative, stains purple with Giemsa, red with Macchiavellos stain, black or dark brown by Levaditi's technique, and light blue with hematoxylin and eosin (TIMONEY et al., 1992; GORHAM & FOREYT, 1998). In lymph nodes, *N. helminthoeca* survives freezing at -20 ºC for 31-158 days; is viable in leukocytes at 4.5 ºC and 52.5 ºC for 48 hours and 2 minutes respectively (GORHAM & FOREYT, 1998); and can be maintained in cell culture for at least three months (RIKIHISA et al., 1991).

With the new reclassification of *Proteobacteria,* the order *Rickettsiales* contains only two families *Rickettsiaceae* and *Anaplasmataceae* (DUMLER et al., 2005). The family *Anaplasmataceae* is composed of four genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* (DUMLER et al., 2001). All members of this family are obligate intracellular organisms that replicate within an enclosed vacuole derived from the host cell (RIKIHISA, 1991b). By polygenetic sequence analysis of the *rrs* and *gro*ESL genes *N. helminthoeca* is a member of the genus *Neorickettsia*, family *Anaplasmataceae* (DUMLER et al., 2001; RIKIHISA et al. 2005). Phylogenetic analyses of the *rrs* gene sequences have demonstrated that

N. helminthoeca is more than 4.5% different from *N. sennetsu* and *N. risticii* (PRETZMAN et al., 1995; HEADLEY et al., 2006). However, these differences are greater when the sequences of the *rpo*B and *gro*ESL genes are analyzed (TILLARDAT-BISCH et al., 2003; RIKIHISA et al., 2005; HEADLEY et al., 2006). Typical phylogenetic trees based on the alignment of the *rrs*, *rpo*B, and *gro*ESL gene sequences for *Anaplasmataceae* are demonstrated (Figure 1).

1.3 GEOGRAPHICAL DISTRIBUTION

Salmon poisoning disease is endemic to the Northwest Pacific of the United States (GORHAM & FOREYT, 1998). This area (Appendix 1) is delimited by the Sacramento River in northwest California, passing through the western coast of Oregon, to the Olympic Peninsula of southwestern Washington, and extending inland to the Pacific slopes of the Cascade Mountains (SIMMS et al., 1931; MILLEMANN & KNAPP, 1970). The disease is also endemic to British Columbia, Canada (BOOTH et al., 1984; RIKIHISA et al., 2005). Recently cases of SPD were diagnosed in Maringá, Southern Brazil (HEADLEY et al. 2006).

1.4 BIOLOGICAL CYCLE OF *NANOPHYETUS SALMINCOLA*

The digenean trematode *Nanophyetus salmincola*, the vector of SPD, requires three hosts to complete its entire life cycle (Appendix 2). The first intermediate host is the pleurocerid snail, *Oxytrema silicula*, which has only been described in the endemic region of the Northwest Pacific of the US (BENNINGTON & PRATT, 1960; MILLEMANN & KNAPP, 1970). This has been considered as the major factor for the restricted occurrence of SPD only within the specific geographical location (SIMMS et al., 1931; RIKIHISA et al; 2005). However, this has never been demonstrated scientifically. The second intermediate hosts are salmonid and a restricted number of non-salmonid fish (GEBHARDT et al., 1966; MILLEMANN & KNAPP, 1970). The definitive hosts are various fishing-eating mammals and birds (BENNINGTON & PRATT, 1960).

The snail (*O. silicula*) is the host of the rediae and cercariae of *N. salmincola*; mature rediae (larvae within a sporocyst) are pathogenic to their host and are found in almost all host tissues (PORTER et al., 1967; MILLEMANN & KNAPP, 1970). Cercariae (free-swimming trematode larvae) when released by the snail into water, penetrate the second intermediate host at any point (but more frequently at the abdominal region), pass through the skin in 30 seconds to 5 minutes, and via the circulatory system, lodge in internal organs (kidneys, heart, liver, and intestines) but mostly in the fleshy part of the tail forming cystic structures (BENNINGTON & PRATT, 1960; BALDWIN et al., 1967; MILLEMANN & KNAPP, 1970). Skin penetration by cercariae is achieved by attachment of the ventral

sucker and body contraction, thereby forming a rigid egg-shaped structure (BALDWIN et al., 1967). Cercariae lose their tails after penetrating the skin and are transformed into metacercariae (tail-less encysted mature larvae) after rupture of the encysted cercariae within the fish (BENNINGTON & PRATT, 1960).

Salmon are infected in fresh water, but maintain the trematode containing *N. helminthoeca* throughout their ocean migration (to salty water), returning to fresh water after up to three years (FARRELL et al., 1964; WEISETH et al., 1974). In cases of fish heavily infected by *N. salmincola*, mortality could be severe, occurring 24 hr after contact with cercariae; death has been associated to the liberation of cercariae-produced proteolytic enzymes, which in high concentrations are toxic to the host (BALDWIN et al., 1967). Adult trematodes develop within the intestine of dogs or fish-eating mammals and birds that have consumed the uncooked metacercariae-infected fish (SCHLEGEL et al., 1968; GORHAM & FOREYT, 1998), or in some cases, smoke-treated processed salmon (FARRELL et al., 1974). Ova from the adult trematode are then released by the parasitized animal and infect the snail, *O. silicula* (JONES et al., 1998). *N. helminthoeca* is maintained throughout the life cycle of the fluke, *N. salmincola*, without being pathogenic to either the first host, or any of the known second intermediate hosts (Table 1). A list of the known definite hosts of *N. salmincola* is provided (Table 2).

1.5 PATHOGENESIS

After ingestion of metacercariae-infected salmonid fish by a susceptible terminal host, the fluke matures within 5-6 days, and the adult stage then attaches deeply within the intestinal mucosa resulting in localized hyperemia, edema, and an influx of inflammatory cells around the parasite (GORHAM & FOREYT, 1998; RIKIHISA et al., 2005). Due to an unknown mechanism, *N. helminthoeca* is then released from the trematode (MILLEMANN & KNAPP, 1970), transferred to resident intestinal monocytes and macrophages, and subsequently via the circulatory and lymphatic systems, arrives and lodges within visceral and somatic lymph nodes, where primary bacterial multiplication occurs (RIKIHISA et al., 2005). Inflammation of Peyer's patches and solitary intestinal lymphoid tissue produces enteritis, which can be hemorrhagic (GORHAM & FOREYT, 1998). *N. helminthoeca* may have some form of tropism for the small intestine (RIKIHISA et al., 2005),

since hemorrhagic enteritis has been demonstrated by oral, intraperitoneal, and intravenous inoculation in dogs (NYBERG et al., 1967). The organism circulates in the blood of infected dogs 8-12 days after infection until death of the animal (RIKIHISA et al., 1991b), which occurs at least 18 days after ingestion of contaminated fish (NYBERG et al., 1967).

N. helminthoeca can also be transmitted to canids experimentally by several ways: injection intraperitoneal of suspensions made from infected blood, spleen, and lymph nodes (CORDY & GORHAM, 1950; BOSMAN et al., 1970); injection of metacercariae (SAKAWA et al., 1973; FOREYT et al., 1987); injection of adult flukes (SIMMS et al., 1931; NYBERG et al., 1967); injection of helminth-infected livers of snail (Philip et al., 1954); the injection of infected eggs of the fluke (NYBERG et al., 1967); and even by aerosol and rectal administration of infected homogenate (BOSMAN et al., 1970).

1.6 CLINICAL MANIFESTATIONS

The normal incubation period after ingestion of parasitized salmon is around 5-7 days (GORHAM & FOREYT, 1998). The first sign of SPD infection is manifested by an increase in body temperature, which normally reaches 40-42 $^{\circ}$C, with a gradual decrease to normal or below normal after the next 4-8 days (CORDY & GORHAM, 1950). A discrete purulent ocular discharge associated with edema of adjacent tissues can be observed after 4-6 days of illness at which time vomiting occurs (JONES et al., 1997). Vomiting is accompanied by marked progressive weight loss, depression, anorexia and the infected dog desperately seeks water (TIMONEY et al., 1992; GORHAM & FOREYT, 1998). Diarrhea, initially blood-tinged and later diffusely impregnated with blood, is progressive and normally observed from the 5th–7th day after infection (TIMONEY et al., 1992). Some dogs may demonstrate gastrointestinal signs that are clinically indistinguishable from those of canine parvovirus (GORHAM & FOREYT, 1998).

Enlarged cervical and prescapular lymph nodes are palpable 5 days after infection (CORDY & GORHAM, 1950). Most untreated dogs die within 6–10 days after the onset of clinical signs, with mortality close to 90% (TIMONEY et al., 1992); less than 5% of dogs infected may recover from SPD, and these demonstrate immunity to reinfection (SIMMS et al., 1931).

1.7 PATHOLOGICAL FINDINGS

The principal gross lesions at necropsy are severe changes to lymphoid tissue. In most cases the mesenteric nodes are at least enlarged to four times their normal size (Figure 2A); somatic lymph nodes are less severely affected (JONES et al., 1997; HEADLEY et al., 2004). Hypertrophic lymph nodes are yellowish, edematous with prominent follicles distributed randomly at the cortex that results in loss of tissue architecture (Figure 2B). Peyer's patches of the intestine are severely hypertrophic. In some cases the lesion can be seen even from the serosal membrane (Figure 2C); within the mucosal surface, hypertrophy of Peyer's patches and lymphoid tissue is severe (Figure 2D). The tonsils of young dogs are enlarged, yellow, with prominent white follicles and, at times, petechiae (FRANK et al., 1974); marked splenic hypertrophy with prominent white follicles are also observed (TIMONEY et al., 1992; JONES et al., 1997). In some cases, the entire intestinal tract is severely hemorrhagic and tinged with mucus (Figure 2E-F). Intussusception of the small intestine may occur; while hepatic ruptures with subsequent peritonitis and hemorrhages of the gallbladder and urinary bladder have been described (JONES et al., 1997).

Microscopic findings are frequently observed in lymphoid tissue and are characterized by hyperplasia of reticuloendothelial cells, depletion of small lymphocytes, foci of necrosis associated with elementary bodies of *Neorickettsia* organisms in reticuloendothelial cells (Figure 2G-H). These organisms are readily demonstrated by Giemsa or Machiavello's staining, and occur within the sinus as well as the parenchyma of hyperplastic lymphoid tissue. Flukes of *N. salmincola* may be observed deep within the intestinal villi or in duodenal glands, without any severe inflammatory reaction, other than a discrete influx of neutrophils, lymphocytes, and plasma cells (CORDY & GORHAM, 1950; TIMONEY et al., 1992; JONES et al., 1997). Other microscopic lesions described include non-suppurative leptomeningitis or meningitis and centrilobular hepatic fatty degeneration (TIMONEY et al., 1992; GORHAM & FOREYT, 1998).

1.8 DIAGNOSIS

A diagnosis of *N. salmincola* infestation is based on the finding of characteristic operculated eggs of the trematode in dog feces 5-8 days after infection, or identifying adult flukes within the intestine (JONES et al., 1997; GORHAM & FOREYT, 1998). Fluke eggs and not the trematode are more frequently observed in dogs with clinical manifestations of SPD (BOOTH et al., 1974). Eggs can be identified by direct smears (GORHAM & FOREYT, 1998) or by a washing-sedimentation technique (BOSMAN et al., 1970). However, diagnosis of the neorickettsial disease cannot be based exclusively on these findings; since trematode infection does not necessarily imply neorickettsial infection and dogs already recovered from the neorickettsial disease may be reinfected by the fluke (GORHAM & FOREYT, 1998).

Diagnosis and confirmation of the neorickettsial disease should be based on: 1) direct microscopic identification of characteristic intracytoplasmic elementary neorickettsial bodies in reticuloendothelial cells stained by Giemsa or the Macchiavellos method obtained from fluid aspirate of lymph nodes or from necropsy specimens (JONES et al., 1997; GORHAM & FOREYT, 1998; HEADLEY et al., 2004); 2) isolation and culture of *N. helminthoeca* in DH82 cells (RIKIHISA et al., 1991); 3) serology using immunofluorescence or Western blotting (KITAO et al., 1973; RIKIHISA, 1991); and/or 4) identification and sequencing of *N. helminthoeca* by PCR using a combination of primers based on the *rrs*, *rpo*B, and *gro*ESL (PRETZMAN et al., 1995; HEADLEY et al., 2006). However, most methods cited in items 2-4 are very costly, require highly specific equipment, and are more frequently used for research. Hematological and biochemical findings are nonspecific for SPD; however, the most consistent findings observed are: thrombocytopenia, lymphopenia, eosinophilia, increased alkaline phosphatase values, and a reduction of serum albumin (GORHAM & FOREYT, 1998).

1.9 THERAPY AND PREVENTION

All canids with SPD should be hospitalized, receive supportive therapy, and be carefully monitored. Dehydration due to vomiting and diarrhea can be controlled by

appropriate administration of isotonic electrolytes; blood transfusion could be implemented in cases of extreme hemorrhage (GORHAM & FOREYT, 1998). Effective control of neorickettsial disease in sick dogs has been reported using oral or intravenous administrations of sulfonamide, oxytetracycline, penicillin, or chloramphenicol (CORDY & GORHAM, 1950; PHILIP, 1954); however, more recent studies of related *Neorickettsia* species suggest that members of this genus are resistant to many antimicrobials, including erythromycin, penicillin, and chloramphenicol (BROUQUI & RAOULT, 1991; RIKIHISA & JIANG, 1988). Thus, the preferred drug for treatment is oxytetracycline administered at 7 mg/kg, IV, 8/8 hours, over 3-5 days (GORHAM & FOREYT, 1998). Praziquantel is highly effective to control the fluke and more efficient results are obtained when administered (via oral or subcutaneous) in a single dose of 10-30 mg/kg (FOREYT & GORHAM, 1988).

There are no known vaccines for SPD, so prevention must be aimed at the elimination of susceptible contaminated fish given raw, uncooked, or inadequately boiled from the diet of canids. Freezing suspected fish at -20ºC during at least 24 hours or thoroughly cooking will destroy the metacercariae and the organism (GORHAM & FOREYT, 1998). Smoked fish has already been known not to destroy *N. helminthoeca* (FARRELL et al., 1974).

1.10 PUBLIC HEALTH CONCERNS

The exact zoonotic potential of *N. helminthoeca* or neorickettsial disease on public health is unknown, so epidemiological studies should be done in humans living in areas where these diseases are endemic to evaluate the impact of these commensalism organisms relative to the possibility of producing direct or indirect disease processes. The only recognized neorickettsial organism that produces clinical disease in humans is *N. sennetsu* (Sennetsu fever described in Japan and Malaysia) for which metacercariae that parasitize grey mullet fish (*Mugil cephalus*) are vectors, but the organism has not been isolated from the trematode (RIKIHISA et al., 2005).

N. risticii is the causative agent of Potomac Horse Fever (PHF) also known as equine monocytic ehrlichiosis, was initially described adjacent to the Potomac River in Maryland and Virginia, USA (HOLLAND et al., 1985). PHF has since been described in 43 of the United States, as well as in Canada, France, Italy, Venezuela, India, Australia

(RIKIHISA et al., 2005), and Brazil (DUTRA et al. 2001; COIMBRA et al., 2005). Cats and dogs are probably less frequently infected than horses, since horses tend to graze and drink more frequently from standing water than these house hold pets (COHN 2003). The bacterium is found in trematodes of the family *Letichodendoriidae* which in turns infects *Pleuroceridae* snails (RIKIHISA et al., 2005). Although not entirely elucidated, the biological cycle may include several fluke species that use river snails (first intermediate host), aquatic insects (the second intermediate host), and insectivorous birds and/or bats (the definitive hosts). Horses are infected while grazing on contaminated pasture or by accidental ingestion/drinking of contaminated aquatic insects, snails, and encysted metacercarie (COHN, 2003; RIKIHISA et al., 2005); bats and a large variety of larvae-eating insects are considered as potential host of *N. risticii* (PUSTERLA et al., 2000; GIBSON et al., 2005). Although PHF has not been proven to be a zoonosis, the global distribution of insects involved in the transmission of this disease associated with the high degree of humans in contact with horses, cats, dogs, and insects, raises the risk substantially.

However, the trematode of SPD has already induced a gastrointestinal disease-syndrome (nanophyetiasis) in humans due to the ingestion of uncooked, partially cooked or smoked salmon and raw eggs of steel-head trout (EASTBURN et al., 1987), and accidental transmission of *Nanophyetus salmincola* during the handling of contaminated salmon has also been reported (HARRELL & DEARDOFF, 1989). PHILIP (1954) injected himself with this organism by eating contaminated salmon. Although the clinical manifestations of SPD did not develop, it is unknown what parasite burden is necessary to produce SPD symptoms or signs in humans. Further, four monkeys experimentally infected with contaminated *N. salmincola* did not present clinical manifestation of SPD, but few eggs of the fluke were found in the stool of two of these animals (KARR & WONG, 1974). Additionally, *N. salmincola schikhobalowi* a closely related endemic intestinal parasite of humans in Siberia has reportedly produced more than 90% parasitism in humans without corresponding manifestation of neorickettsial disease, probably because this fluke does not harbor any neorickettsial agent (MILLEMANN & KNAP, 1970). Since these historical cases, there are no other published reports of human nanophyetiasis in the Americas, but this does not necessarily imply that the disease does not exist. Nevertheless, *Nanophyetus salmincola* is considered as a potential zoonosis (ACHA & SZYFRES, 1986) for which neorickettsiosis should be considered a risk. Furthermore, in Brazil the habit of consuming meals prepared from raw salmon has increased drastically over the last years, thereby increasing the risk of human neorickettsiosis.

Additionally, *Neorickettsia* sp. are known to coexist within their trematode hosts, are maintained unmodified throughout the biological cycle of the trematode and are readily transferred to mammalian monocytes, so there is a great possibility that humans infected by these trematodes could be co-infected with neorickettsial organisms.

1.11 CONCLUSION

N. helminthoeca produces salmon poisoning disease in canids, which was previously described only in the USA and Canada; cases have been recently described in Brazil. The digenean trematode, *Nanophyetus salmincola*, is the vector of SPD and maintains the organism throughout its entire lifecycle. This life cycle includes a river snail, *Oxytrema silicula*, fish, and fish-eating mammals. The entire list of secondary and definite hosts and the mechanisms of infection to the mammalian host cells are unknown; there is no known vaccine against this agent. Further, because *N. helminthoeca* and *N. salmincola* can produce coinfections within the same mammalian host more, attention should be given to the potential for neorickettsial infections in mammals, including humans.

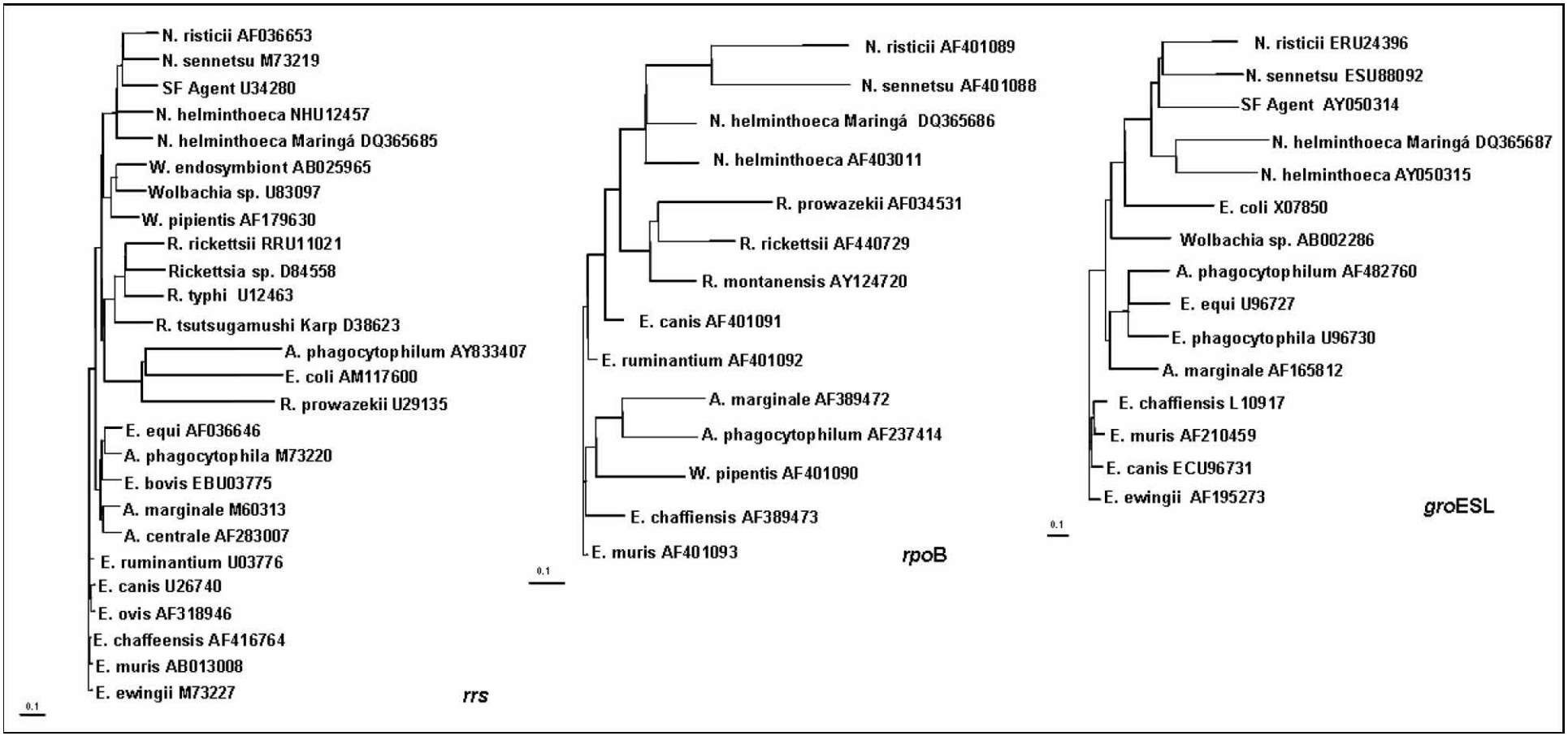

Figure 1. Phylogenetic trees showing the relationship of *Anaplasmataceae* based on the *rrs*, *rpo*B, and *gro*ESL gene sequences. Trees were constructed by the Vector NTI Advance™ 10 Software (bars represent substitutions per 1,000 bp; sequence accessions numbers are given after sequence).

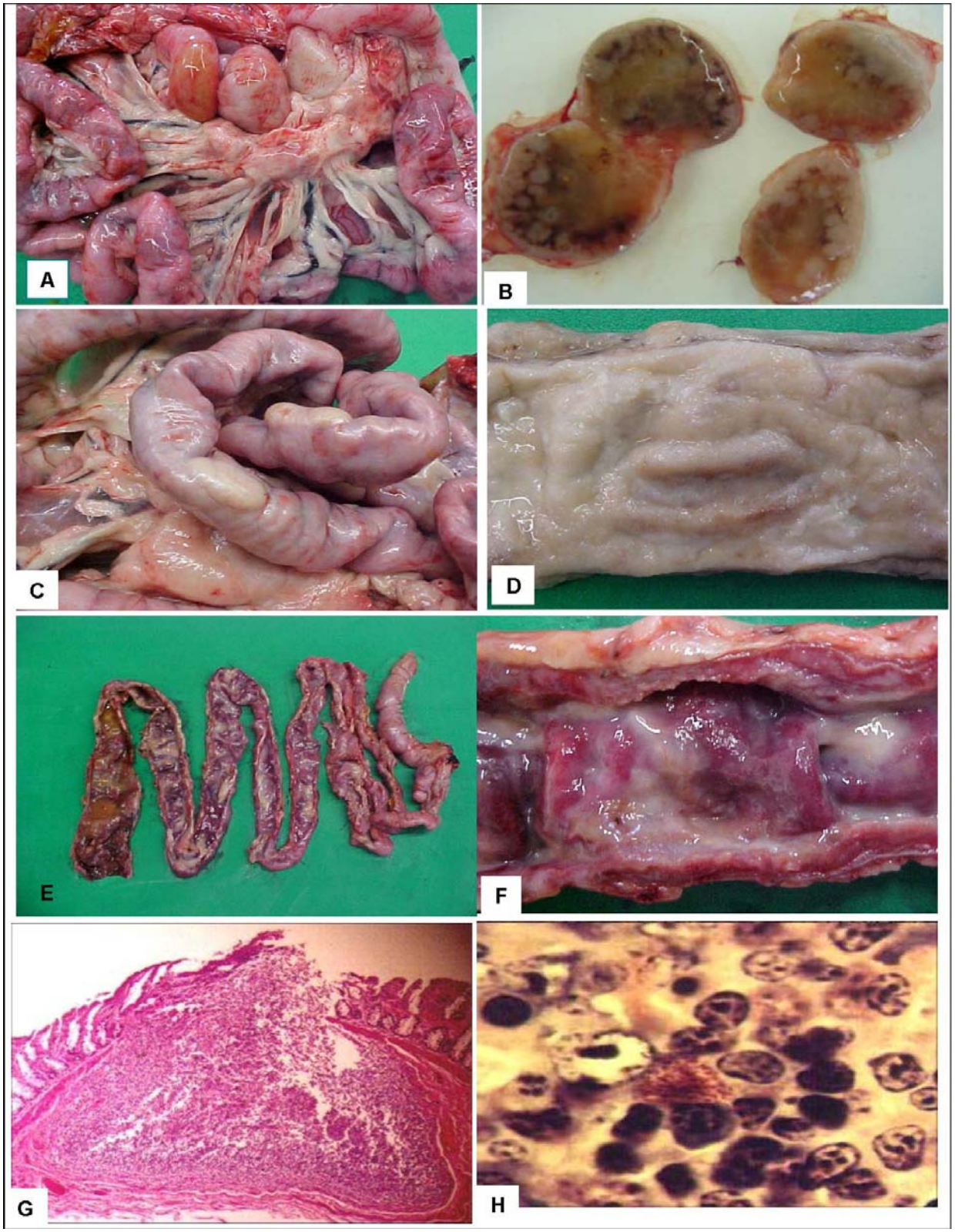

Figure 2. Composite photography. Salmon poisoning disease of dogs. Gross images (A-F); observe marked hypertrophy of mesenteric lymph node (A) and randomly distributed white cortical follicles of the sectioned node (B). Hypertrophy of Peyer's patches can be observed from the serosal (C) and mucosal membranes of the intestine (D). Severe hemorrhage throughout the intestinal tract (E,F). Photomicrograph; there is severe proliferation of lymphoid tissue within the intestine. H&E, Obj. 4x (G). Observe hyperplasia of lymphocytes and elementary intracytoplasmic neorickettsial bodies in a reticuloendothelial cell. Giemsa stain, Obj. 100x (H).

Table 1 Second intermediate hosts for *Nanophyetus salmincola,* the vector of salmon poisoning disease[1].

HOST	TYPE OF INFECTION[‡]		REFERENCES
	N	E	
Salmonidae			
Oncorhynchus tshawytscha (Chinook salmon)	+	+	Donham et al., 1926; Bennington & Pratt, 1960; Foreyt et al., 1987.
O. kisutch (Coho salmon)	+	+	Donham et al., 1926; Baldwin et al., 1967; Farrell et al., 1967; Harrell & Deardorff, 1989.
O. *keta* (Chum salmon)	+	-	Simms et al., 1931.
O. merka (Kokanee)		+	Baldwin et al., 1967.
Salmo gairdneri (Rainbow trout)	+	+	Simms et al., 1931; Baldwin et al., 1967.
S. clarki clarki (Cut throat trout)	+	+	Simms et al., 1931; Baldwin et al., 1967.
S. clarki lewisi (Montana black-spotted cut throat trout)	-	+	Baldwin et al., 1967.
S. clarki henshawi (Lahontan Cut throat trout)	-	+	Baldwin et al., 1967.
S. solar (Atlantic salmon)	-	+	Gebhardt et al. 1966.
S. truta (Brown trout)	-	+	Gebhardt et al. 1966
Salvelinus fontinalis (Brook trout)	+	+	Simms et al. 1931; Gebhardt et al. 1966.
S. namaycush (Lake trout)	-	+	Gebhardt et al. 1966.
Petromyzontidae			
Lampetra richardsoni (Western brook lamprey)	+	+	Gebhardt et al. 1966.
L. tridentata (Pacific lamprey)	+	-	Gebhardt et al. 1966.
Cottidae			
Cottus perplexus (Reticulate sculpin)	+	+	Gebhardt et al. 1966.
Cyprinidae			
Carassius auratus (Goldfish)	-	+	Bennington & Pratt, 1960; Gebhardt et al. 1966.
Rhinichthys osculus (Speckled dace)	-	+	Bennington & Pratt, 1960.
Richardsonius balteatus (Redside shiner)	+	+	Gebhardt et al. 1966.
Catostomidae			
Catostomus macrocheilus (Large scale sucker)	-	+	Gebhardt et al. 1966.
Centrarchidae			
Lepomis macrochirus (Bluegill)	-	+	Gebhardt et al. 1966.
Gasterosteidae			
Gasterosteus a. aculeatus (Three spine stickback)	-	+	Gebhardt et al. 1966.
Gasterosteus a. microcephalus (Three spine stickback)	-	+	Gebhardt et al. 1966.
Poeciliidae			
Gambusia affinis (Mosquito fish)	-	+	Gebhardt et al. 1966.
Percidae			
Perca flavescens (Yellow perch)	-	0	Gebhardt et al. 1966.
Ambystomidae			
Dicamptodon ensatus (Pacific giant salamander)	+	-	Gebhardt et al. 1966.

[1] Adapted from MILLEMANN & KNAPP, 1970.
[‡] N, natural; E, experimental; negative sign, information unavailable; 0, failure to achieve infection.

Table 2. Definite hosts for *Nanophyetus salmincola*, the vector of salmon poisoning disease[1].

TYPE OF INFECTION		REFERENCES
Natural	**Experimental**	
Man	Man	Philip, 1958; Harrell and Thomas, 1986; Eastburn et al; 1987.
Ardea herodias (Great blue heron) *Lophodytes cumullatus* (Hooded merganser) *Mustela erminea* (Short tail weasel) *Lutra canadensis* (River otter)	*Didelphis marsupialis* (Opossum) *Spilogale putorius* (Spotted skunk)	Schlegel et al., 1968.
Canis familiaris (Domestic dog)	*Canis familiaris* (Domestic dog)	Simms et al., 1931; Schlegel et al., 1968; Cordy & Gorham, 1950; Schlegel et al., 1968.
Vulpes fulca (Red fox)	*Vulpes fulca* (Red fox)	Donham et al., 1926; Simms et al., 1931; Cordy & Gorham, 1950; Schlegel et al., 1968.
Rattus norvegicus (Norway rat)	*Rattus norvegicus* (Norway rat)	Simms et al., 1931; Schlegel et al., 1968.
Procyon lotor (Raccoon)	*Procyon lotor* (Raccoon)	Simms et al., 1931; Bennington & Pratt, 1960; Schlegel et al., 1968.
Felis domesticus (Domestic cat)	*Felis domesticus* (Domestic cat)	Simms et al., 1931; Schlegel et al., 1968.
Canis latrans (Coyote)	*Canis latrans* (Coyote)	Donham et al., 1926; Simms et al., 1931; Schlegel et al., 1968; Foreyt et al., 1987.
Alopex latrans (Artic fox)	*Alopex latrans* (Artic fox)	Simms et al., 1931.
Lynx rufus (Bobcat)	*Lynx rufus* (Bobcat)	Simms et al., 1931; Schlegel et al., 1968.
	Cavia porcellus (Guinea pig)	Simms et al., 1931.
	Mesocricetus auratus (Golden hamster)	Philip 1959; Bennington & Pratt, 1960.
	Mus musculus (White mouse)	Philip 1954a.
	Neotoma sp. (Wood rat)	Bennington & Pratt, 1960.
	Macaca mulatta (Rhesus monkey) *M. nemestrina* (Pigtailed macaque) *Cercocebus atys* (Sooty mangabeys)	Karr & Wong, 1974.
Mustela vison (Mink)		Donham et al., 1926; Simms et al., 1931; Schlegel et al., 1968.

[1] Adapted from SCHLEGEL et al., 1968.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Determinar a etiologia e descrever a patologia associada às alterações linfóides induzidas por organismos intracitoplasmáticos encontrados em cães durante a necropsia de rotina na cidade de Maringá, Paraná, Brasil.

2.2 OBJETIVOS ESPECÍFICOS

a) Descrever o quadro anatomo-patológico observado em cães associado aos organismos intracitoplasmáticos.
b) Caracterizar a etiologia dos organismos intracitoplasmáticos observados nos tecidos linfóides de cães utilizando as técnicas de biologia molecular.
c) Determinar o grau de similaridade entre o agente encontrado nesses animais com a *Neorickettsia helminthoeca*.

3 ARTIGOS PARA PUBLICAÇÃO

3.1 SUSPECTED CASES OF *NEORICKETTSIA*-LIKE ORGANISMS IN BRAZILIAN DOGS[1]

[1] Published in **Annals New York Academy of Sciences**. v. 1026, p. 79–83, 2004.

Suspected Cases of *Neorickettsia*-like Organisms in Brazilian Dogs[2]

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[2] Published in **Annals New York Academy of Sciences**. v. 1026, p. 79–83, 2004.

Abstract

Preliminary investigative findings of gross and histopathological lesions consistent with salmon poisoning disease in 10 dogs from Southern Brazil are described. Lesions were restricted to the spleen, lymph nodes and intestinal lymphoid tissues. Grossly, there was marked hyperplasia of mesenteric lymph nodes and Peyer's patches. Microscopic alterations were characterized by diffuse hyperplasia of intestinal lymphoid tissues and Peyer's patches. Intracytoplasmic organisms consistent with *Neorickettsia helminthoeca* were demonstrated by Giemsa stain in reticuloendothelial cells of the intestine, spleen, Peyer's patches and lymph nodes. We have named this organism *Neorickettsia helminthoeca*-like because of its marked similarity with the agent described in the United States.

Keywords: *Neorickettsia helminthoeca*, dogs, pathology, parasitology.

Introduction

Salmon poisoning disease (SPD) is a granulomatous enterocolitis of dogs and foxes caused by *Neorickettsia helminthoeca, a* coccoid or cocobacillary rickettsia, that occurs in specific geographical locations of the United States[1,2]. The apparent limitation of SPD to the United States has been directly related to the restricted presence of the intermediate host, a pleurocerid snail, *Oxytrema silicula*[3] . However, cases of SPD have been described elsewhere[4].

Animals infected by SPD are severely emaciated, anorexic, depressed with enlarged lymph nodes and normally die within 14 days post infection[3]. Principal gross lesions are marked hypertrophy of the tonsils, mesenteric lymph nodes, Peyer's patches, intestinal lymphoid tissue, moderate splenomegaly, and hemorrhagic enteritis[5,6]. Microscopically, SPD is characterized by severe and diffused hyperplasia of reticluoendothelial cells and lymphocytic depletion, principally in mesenteric lymph nodes[1,5,6]. Macrophages exhibit large quantities of elementary intracytoplasmic rickettsial organisms demonstrable by Giesma staining[3,5,6].

This report describes the preliminary investigative results of ten Brazilian dogs with intestinal and lymphoid alterations comparable to salmon poisoning disease.

Materials and methods

Five mongrels (3 males, 2 females) and five Beagles (2 females, 3 males), age ranging between two to four years were sacrificed; routine necropsy was preformed soon after death. Tissues were fixed in 10% formalin solution and routinely processed for histopathological evaluation. Selected specimens were stained by Giemsa in order to determine the presence or absence of intracytoplasmic organisms possibly consistent with *N. helminthoeca*.

Results

All dogs were in good body condition and presented similar gross lesions. Grossly, pre-scapular, mesenteric, and axial lymph nodes were markedly enlarged. Sectioned surface revealed prominent white cortical follicles; discrete to moderate edema was observed in some lymph nodes. All dogs demonstrated severe and multifocal hypertrophy of Peyer's patches and intestinal lymphoid tissue (Figure 1.1). Moderate splenomegaly was observed in two mongrels; in these cases, prominent white follicles were observed at the sectioned splenic surface. Diffuse hemorrhagic colitis was observed in one mongrel.

Microscopic intestinal lesions were characterized by severe and diffuse hyperplasia of lymphoid tissue; Peyer's patches were markedly hyperplasic (Figure 1.2). Mesenteric lymph nodes demonstrated marked cortical depletion, with sparing of few germinating centers. Similar microscopic alterations were observed in the spleen. Small intracytoplasmic organisms comparable to *Neorickettsia helminthoeca*[1,5,6,] demonstrated by Giemsa stain were observed in macrophages within Peyer's patches, intestinal glands, germinating centers of mesenteric lymph nodes, and the splenic corpuscles. Microscopic sections of the large intestine of one Beagle revealed several thick-walled, 0.38 to 0.51µm in length and 0.11 to 0.12µm in width, trematodes comparable to *Ascocotyle* (*Phagicola*) *arnaldoi*[7] (Figure 1.3).

Discussionand conclusions

The intracytoplasmic organisms observed in the present report are morphologically similar and produced pathological alterations characteristic of *N. helminthoeca*[1,3,5,6]. Although SPD has been considered restricted to specific geographic locations of the USA[1,3], cases have been diagnosed elsewhere[4], so there exists a possibility of having SPD-like disease in other countries.

The feeding of crude fish to dogs is not a common practice in Brazil, so the biological cycle of *N.*

helminthoeca does not corresponds exactly to the intracellular organism observed in this investigation. However, we believe that the intracytoplasmic organisms observed in the present report may be closely related to *N. helminthoeca.* Three members (*Phagicola angrense, P. angeloi*, and *P. arnaldoi*) of the genus *Ascocotyle* (*Phagicola*) have been previously described in Brazil[7]. We strongly believe that the trematode observed in this report is *Ascocotyle* (*Phagicola*) *arnaldoi*; however we were unable to obtain live specimens of the parasite to fully characterize the trematode. This trematode, like *Nanophyetus salmincola* that is associated with SPD, requires two intermediate hosts (specific snails and fishes) to complete its life cycle in the intestine of the definite host[8].

Immunohistochemical and molecular biology techniques are being currently implemented to characterize the etiologic agent. Epidemiological studies are being undertaken to determine the source of infection, the intermediate hosts, and the biological cycle of this *N. helminthoeca*-like organism in Brazil. We have named the intracellular organism *Neorickettsia helminthoeca*-like because of its marked morphological similarity with the organism described in the United States and the similar pathological intestinal and lymphoid alterations to which the SPD agent has been associated[1,3,5,6].

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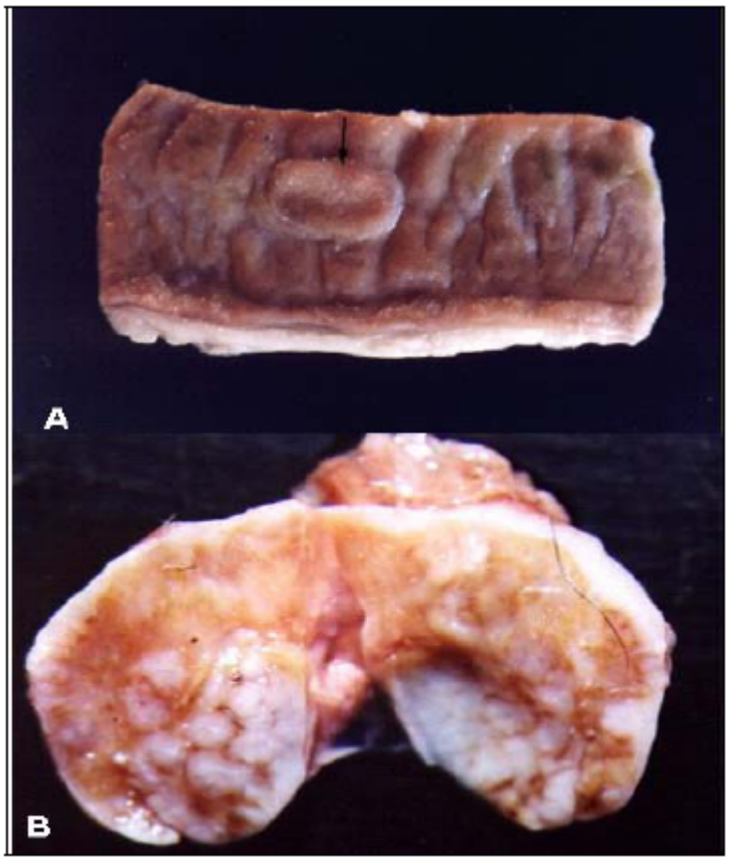

Figure 1.1. Composite microphotography. Salmon poisoning disease: gross lesions in a dog, Beagle, small intestine (A). There is marked hypertrophy of Peyer´s patches (arrow). B. Sectioned mesenteric lymph node; observe white follicles located principally at the cortex.

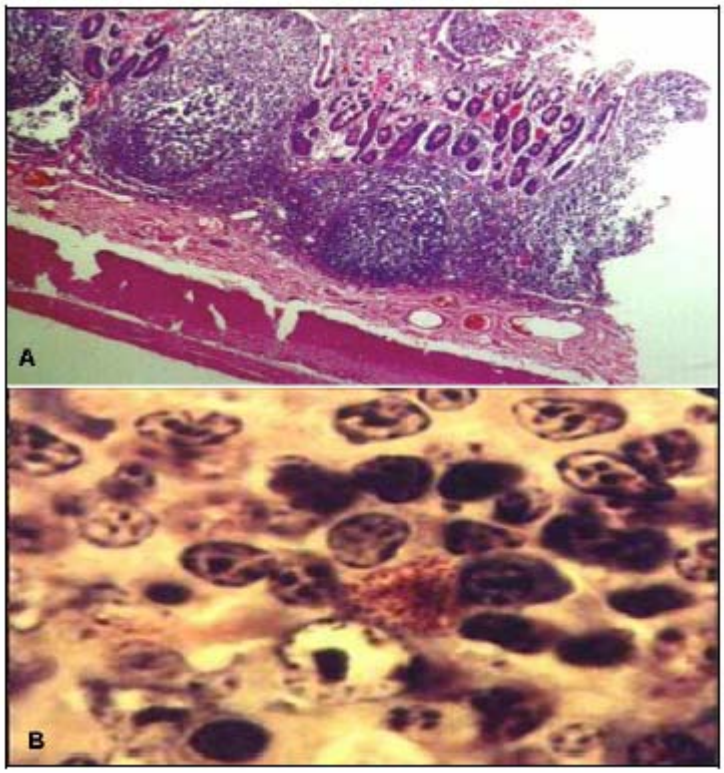

Figure 1.2. Composite microphotography. Microscopic lesions in the small intestine of a dog, Beagle. A, observe marked proliferation of lymphoid tissue (Obj. 10x; HE); B, intracytoplasmic organisms packed within reticuloendothelial cell (Obj. 100x: Giemsa).

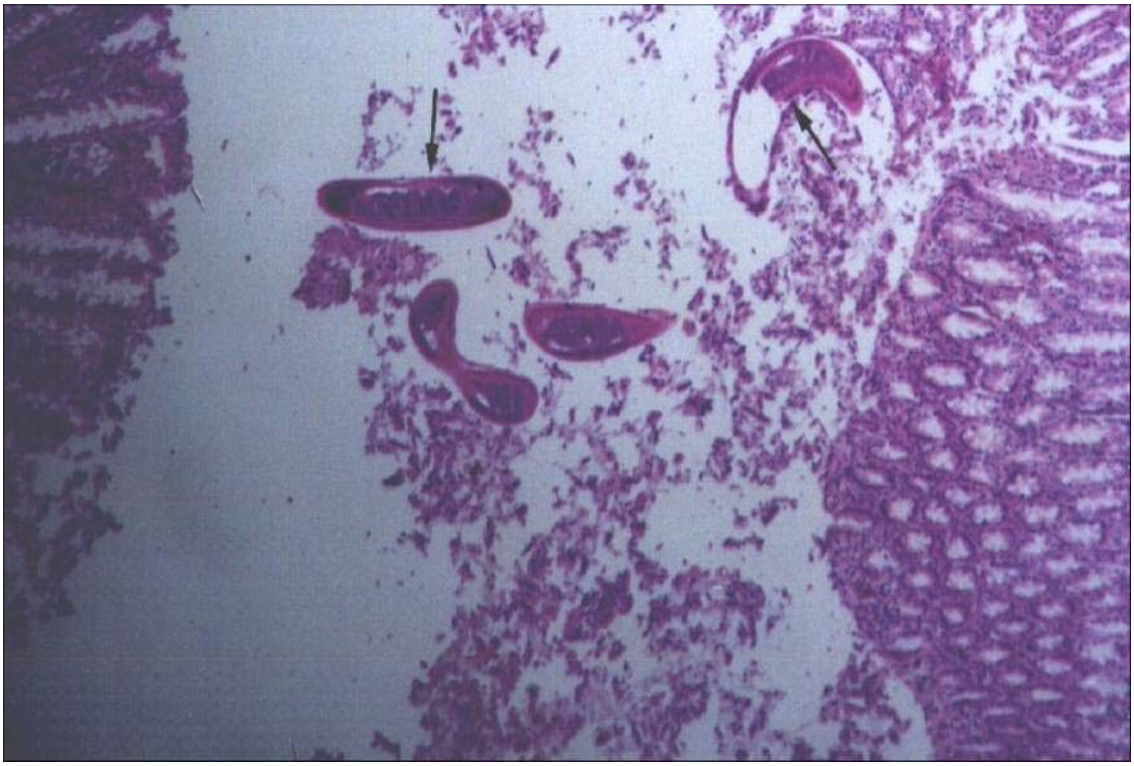

Figure 1.3. Composite microphotography. Salmon poisoning disease. Dog, Beagle, large intestine. There are trematoeds (arrows) within the lumen (Obj. 10x, HE).

3.2 *NEORICKETTSIA HELMINTHOECA* IN A BRAZILIAN DOG [1]

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To the editor: *Neorickettsia helminthoeca* is the cause of salmon poisoning disease (SPD) of canids, described only in the Northwest Pacific States of the USA and Canada (1). The trematode *Nanophyetus salmincola* is the vector of SPD and maintains the bacterium throughout its entire life cycle (2, 3), which includes a river snail (*Oxytrema silicula*), fish (salmonid and trout), and canids (3). We describe the first cases of *N. helminthoeca* in dogs beyond the known endemic region of SPD.

From 2001 to 2005, 20 adult dogs (5 mongrels and 15 Beagles) of both sexes demonstrated pathological lesions characteristic of SPD (4). All Beagles were born in the coastal city of Florianópolis, Santa Catarina; and later transferred to the city of Maringá, Paraná, Brazil. Selected tissues (lymph nodes, spleen, liver, and intestine) from ten Beagles were harvested aseptically at necropsy in Maringá. All laboratory procedures were performed at The Johns Hopkins Medical Institutions (JHMI), Baltimore, MD, USA.

Genomic DNA was extracted from tissues by using the QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). Pure DNA of *N. helminthoeca* and *Anaplasma phagocytophilum* maintained in DH82 and HL-60 cells, respectively, were used as positive controls. Nuclease-free water (Promega Corporation, Madison, WI, USA) was the negative control.

Neorickettsia spp. 16S ribosomal RNA (*rrs*)-specific primers and *N. helminthoeca*-specific primers based on the RNA polymerase *β*-subunit, *rpo*B (5) and the heat shock protein *gro*ESL (6,7) genes were designed; established citrate synthase gene (*glt*A) primers (8) were also used. Two rounds of PCR were used for to maximize sensitivity, and great care was taken to minimize contamination. All PCR products were separated by electrophoresis in 1% agarose gels. PCR product clean-up was performed (Qiagen Inc., Valencia, CA, USA) before amplicons were cloned (pGEM®-T and pGEM®-T Easy Vector Systems, Promega Corporation). Plasmids obtained were recovered and sequencing was done at the DNA Analysis Facility, JHMI. The Maringá sequences obtained were compared with those deposited in the GenBank by using the program BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees, sequence alignments, and identity tables were created by using the Vector NTI Advance™ 10 Software (Invitrogen Corporation, Carlsbad, CA, USA). GenBank accession numbers of *Anaplasmataceae* used in this study and their phylogenetic relationships are shown (Figure 2.1).

Two dogs (N40-05, mesenteric lymph node, Maringá 1, and N20-04, Peyer's patch, Maringá 2) contained *Neorickettsia* spp. *rrs*, *rpo*B, and/or *gro*ESL genes. Both samples

produced sequences for the *Neorickettsia* spp. *rrs* gene. However, *N. helminthoeca rpo*B and *gro*ESL sequences were obtained from only dog 1. All dogs were negative using the *glt*A gene primers. The *rrs* DNA sequences from these 2 Maringá dogs and *N. helminthoeca* were 100% identical. A 100% sequence identity was observed between dog 1 and *N. helminthoeca* for the *gro*ESL and *rpo*B genes. All negative controls yielded no product, further confirming the lack of amplicons contamination.

This study demonstrates that 2 Brazilian dogs from the city of Maringá, Paraná, with characteristic pathological lesions of SPD (2,9), were infected with a *Neorickettsia* spp. Moreover, sequences obtained from dog 1 were identical to those of *N. helminthoeca* for the *rrs*, *gro*ESL, and *rpo*B genes, confirming our theory of infection by this organism (4). To the authors' knowledge, this is the first description of this organism beyond the known geographical area of SPD. The organism identified in Brazil has been called *N. helminthoeca* Maringá strain due to the geographical location of the dogs.

Owing to the difficulty in recovering DNA from these tissue samples and the need to use a highly efficient PCR targeting small DNA regions, the sequences obtained for *N. helminthoeca* Maringá dog 1 were comparatively short (112 bp *rrs*; 92 bp *gro*ESL; 143 bp *rpo*B) relative to the sequences deposited in GenBank (*rrs,* 1453 bp; *gro*ESL*,* 1914 bp; *rpo*B, 464). Therefore, more work must be done to obtain larger sequences for more accurate genotypic comparisons. Furthermore, we propose that further study will allow recovery of the pathogen from other dogs so that comparative biological analyses can be initiated. The method of infection-transmission in these cases has not been elucidated. Epidemiological studies are being undertaken to determine the biological life cycle of the organisms associated with this bacterium in Brazil and South America.

Understanding of the biology of infections by *Neorickettsia* spp. has expanded dramatically in recent years, incriminating highly complex life cycles that involve mature and immature helminths, aquatic insects, several different mammals, including even bats (10). While SPD is caused by *Neorickettsia helminthoeca*, infections by other *Neorickettsia* spp., including *N. risticii* (Potomac horse fever) and *N. sennetsu* (sennetsu fever), illustrate the broad potential of these widely distributed species to infect and cause disease in mammals and man, alike. The detection of *N. helminthoeca* in dogs from Brazil further extends the range of this species and warrants a broad search for infections and spectrum of disease caused by neorickettsial organisms in animals and man.

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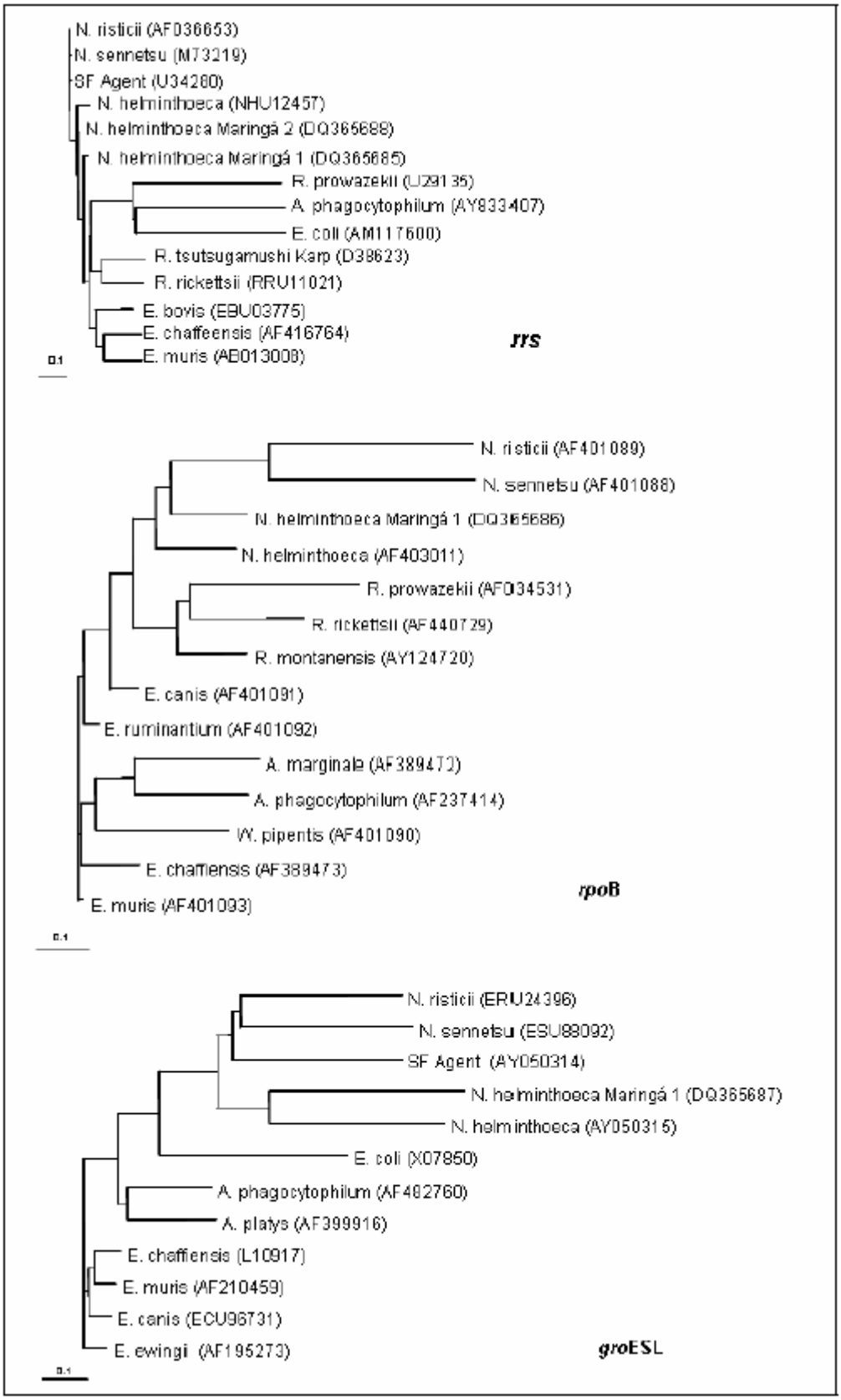

Figure 2.1. Neighbor-joining phylogenetic trees based on the of *Anaplasmataceae rrs*, *rpo*B, and *gro*ESL gene sequences of *Anaplasmataceae* family. Trees were constructed by the Vector NTI Advance™ 10 Software (bars represent substitutions per 1,000 bp; GenBank sequence accession numbers are given in parenthesis).

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3.3 MOLECULAR CHARACTERIZATION OF *NEORICKETTSIA HELMINTHOECA* IN BRAZILIAN DOGS BY PCR ANALYSIS AND SEQUENCING OF THE 16S RIBOSOMAL RNA (*RRS*), RNA POLYMERASE *B*-SUBUNIT (*RPO*B), AND THE HEAT SHOCK PROTEIN (*GRO*ESL) GENES[2].

[2] Part of the PhD thesis presented by the first author at the Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

Molecular characterization of *Neorickettsia helminthoeca* in Brazilian dogs by PCR analysis and sequencing of the 16S ribosomal RNA (*rrs*), RNA polymerase *β*-subunit (*rpo*B), and the heat shock protein (*gro*ESL) genes[3].

Characterization of *Neorickettsia helminthoeca* in Brazilian dogs.

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Abstract

Neorickettsia helminthoeca is the cause of salmon poisoning disease (SPD) described in canids within the endemic region of the Northwest Pacific States of the USA, and Canada. Cases of *N. helminthoeca* were diagnosed in two dogs (Maringá 1, mesenteric lymph node; Maringá 2, Peyer's patch) from the city of Maringá, Paraná, Brazil, that demonstrated gross and histological lesions consistent with those described in SPD. *N. helminthoeca* was confirmed by PCR using primers targeted at the *Neorickettsia* spp. 16S ribosomal RNA (*rrs*), and at the RNA polymerase *β*-subunit (*rpo*B*)* and the heat shock protein (*gro*ESL) genes of *N. helminthoeca*. Samples from both dogs produced DNA sequences that demonstrated a 100% sequence identity (Maringá 1, 112 bp; Maringá 2, 107 bp) when compared to that of *N. helminthoeca* for the *rrs* gene. Moreover, the DNA nucleotide sequences obtained from the Maringá 1 dog demonstrated a 100% sequence identity relative to those of *N. helminthoeca* deposited in GenBank for the *rrs* (112 bp), *rpo*B (143), and *gro*ESL (92 bp) genes. **Key words:** *Neorickettsia helminthoeca,* salmon poisoning disease, molecular biology, pathology, dogs.

Introduction

Neorickettsia helminthoeca, an intracellular obligate bacterium of monocytes, is the cause of salmon poisoning disease (SPD) of canids observed only in the Northwest Pacific States of the USA, and Canada (Dumler et al 2005). The pathology of SPD was revealed in 1950 (Cordy & Gorham, 1950), and the agent was described by Philip et al (1954a). *N. helminthoeca* is the first recognized obligatory helminth-borne pathogenic agent described in bacteriology (Philip et al (1954a; 1954b). Further, SPD is a misnomer that was recognized by white settlers in the 19th Century in Pacific Northwest and so named because dogs became acutely sick and died after eating salmon (Philip, 1955).

Clinically, SPD is characterized by fever, depression, bloody diarrhea, severe body wasting, and generalized lymphadenopathy (Cordy & Gorham, 1950; Jones et al 1997; Gorham & Foreyt, 1998). If not treated, mortality could attain more than 90% of infected dogs (Gorham & Foreyt, 1998). Gross lesions include hypertrophy of mesenteric lymph nodes and intestinal lymphoid tissue, and intestinal hemorrhage (Cordy & Gorham, 1950; Jones et al 1997). Histological alterations are prominent in lymphoid tissue and are characterized by hyperplasia of reticuloendothelial cells, depletion of lymphocytes, focal necrosis, and intestinal hemorrhage (Cordy & Gorham, 1950; Frank et al 1974; Headley et al 2004).

Macrophages within these lesions exhibit large quantities of elementary intracytoplasmic neorickettsial elementary organisms demonstrable by Giesma staining (Cordy & Gorham, 1950; Jones et al 1997).

The vector of SPD is a trematode, *Nanophyetus salmincola,* which maintains the bacterium throughout its entire life cycle (Gorham & Foreyt, 1998; Millemann & Knapp, 1970; Rikihisa, 1991). This cycle includes a river snail (*Oxytrema silicula*), fishes (principally, salmon and trout), and canids (Bennington & Pratt, 1960; Millemann & Knapp, 1970). The snail, *Oxytrema silicula,* is endemic in certain rivers of the Pacific Northwest of the USA which has resulted in the occurrence of SPD in canids only within this specific geographical location (Sims et al 1931; Rikihisa, 1991; Rikihisa et al 2005). Additionally, *N. helminthoeca* does not infect the fish or the snail carrying the fluke (Rikihisa et al 2005).

Phylogenetic studies using the 16S ribosomal RNA, *rrs* (Pretzman et al 1995; Wen et al 1996; Dumler et al 2001), heat shock protein, *gro*ESL (Dumler et al 2001; Rikihisa et al 2004), RNA polymerase *β*-subunit, *rpo*B (Tillardat-Bisch et al 2003), and the citrate synthase, *glt*A (Inokuma et al. 2001) genes have demonstrated that *N. helminthoeca* is closely related to *N. sennetsu, N. risticii,* and the *Stellantchasmus falcatus* Agent (SF Agent). These genes are frequently used to characterize and/or analyze phylogenetic relationships between *Anaplasmataceae*. With the reclassification of *Proteobacteria,* the order *Rickettsiales* contains only two families *Rickettsiaceae* and *Anaplasmataceae* (Dumler et al 2005). The family *Anaplasmataceae* is composed of four genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* (Dumler et al 2001). All members of this family are obligate intracellular organisms that replicate within an enclosed vacuole derived from the host cell (Rikihisa, 1991b).

Recently, 10 dogs with gross and histopathological lesions consistent with those induced by *N. helminthoeca* were observed in the city of Maringá, Paraná, Southern Brazil (Headley et al 2004). Due to close morphological similarities with SPD as described in the Pacific Northwest, the agent was called *N. helminthoeca*-like, and studies were initiated to fully characterize the agent. Consequently, this paper describes the investigations used to identify *N. helminthoeca* in Brazilian dogs.

Materials and methods

Sampling of dogs and DNA extraction. From 2001 to 2005, 20 adult dogs (5 mongrels and 15 Beagles), of both sexes, demonstrated pathological lesions (Fig. 3.1) consistent with those

induced by *N. helminthoeca* (4). All Beagles were born and raised in the coastal city of Florianópolis, Santa Catarina, but spent their adult life in the city of Maringá, Paraná, Brazil. Selected tissues (lymph nodes, spleen, liver, and intestine) from ten Beagles were harvested aseptically at necropsy at the Veterinary Teaching Hospital, Maringá, and maintained at -20 ^{o}C until use. Genomic DNA was extracted from tissues by using the QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA, USA). For controls, DNA was obtained from tissue cultures of pure *N. helminthoeca* and *Anaplasma phagocytophilum* maintained in DH82 and HL-60 cells, respectively. Nuclease-free water (Promega Corporation, Madison, WI, USA) was used as negative control.

PCR amplification of *rrs, rpoB, glt*A, and *groESL* genes of *N. helminthoeca*. The following primers were designed by using PrimerQuest® (http://scitools.idtdna.com/Primerquest) after alignment of these genes for many *Anaplasmataceae*: 1) *Neorickettsia* spp. *rrs* gene-specific primer pairs NeoSH-F (5'-TAGGCCCGCGTTAGATTAGCTTGT-3') and NeoSH-R (5'-TACAACCCAAGGGCCTTCATCACT-3'); 2) *N. helminthoeca rpoB* gene-specific primers (Tilladrt-Bisch et al., 2003) (NH-*rpo*B-F: 5'-TGTCTTCGAAGGCCCAAAGACAGA-3' and NH-*rpo*B-R: 5'- AGAACCGATAGAGCGGGCATGAAT-3'); and 3) *N. helminthoeca gro*ESL gene-specific gene primers (Dumler et al 2001; Rikihisa et al 2004) (NH-*gro*ESL-F: 5'-AGGCTACTTCGCAGGCAAATGAGA-3' and NH-*gro*ESL-R: 5'-CACGCTTCATTCCGCCCTTTAACT-3'). The *N. helminthoeca* primers based on the citrate synthase gene (*glt*A) (Inokuma et al 2004) considered specific for this organism were also used. For PCR, a 50µl final solution containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.1 mM dNTPs, 0.2 µM of each primer, 2.5 U Taq polymerase, and 2µl extracted DNA was used. All PCR products were separated by electrophoresis in 1% agarose gels, the bands were excised, cleaned by using the DNA Extraction from Agarose Gels kit (Qiagen Inc., Valencia, CA, USA), and re-amplified using 5µl of the newly acquired DNA with the same primer pairs, if needed. The PCR protocol is shown (Appendix 3).

Cloning and sequencing of the PCR product. Amplicons from PCR reactions were cloned by using the pGEM® -T and pGEM® -T Easy Vector Systems (Promega Corporation). Transformation reactions were done by using JMI 109 High Efficiency Competent Cells (Promega Corporation). Transformation cultures were placed on plates containing a mixture

of LB broth, ampicillin, IPTG (Promega Corporation), and X-Gal, (Promega Corporation), after which individual cultures were grown in LB broth with ampicillin. Plasmids obtained were recovered by using the GenElute Plasmid Miniprep Kit (Sigma, Saint Louis, Missouri, USA), and digested with the EcoR I restriction enzymes (Promega Corporation). Sequencing was done at the DNA Analysis Facility (Johns Hopkins Medical Institutions).

Computer analyses of DNA sequences. The DNA sequences obtained from these dogs were compared with known sequences deposited in the GenBank nucleotide database by using the program Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic trees, sequence alignments, and identity tables were created based on the comparison between know nucleotides from selected *Anaplasmataceae* members and the obtained nucleotides sequences by using the Vector NTI Advance™ 10 Software (Invitrogen Corporation, CA, USA), which is based on the Neighbor Joining method.

Nucleotide sequence accession numbers. The sequences obtained in this study have been deposited in the GenBank database under the indicated accession numbers: *N. helminthoeca* Maringá strain for the *rrs* gene Dog 1 DQ365685 and Dog 2 DQ365688; *N. helminthoeca* Maringá strain for the *rpo*B-gene (Dog 1), DQ365686; and *N. helminthoeca* Maringá strain dog 1 for the *gro*ESL-gene, DQ365687. The GenBank accession numbers of the *rrs* gene sequences used to construct polygenetic trees and calculate percent identities are: *A. phagocytophilum*, AY833407; *E. bovis,* EBU03775; *E. chaffeensis*, AF416764; *E. coli*, AM117600; *E. muris*, AB013008; *N. helminthoeca*, NHU12457; *N. risticii,* AF036653; *N. sennetsu,* M73219*; R. prowazekii*, U29135; R. rickettsii, RRU11021; *R. tsutsugamushi* Karp, D38623; and the SF Agent, U34280. The GenBank accession numbers of the following *rpo*B genes were used: *A. marginale,* AF389472; *A. phagocytophilum*, AF237414; *E. canis,* AF401091; *E. chaffiensis,* AF389473; *E. muris*, AF401093; *E. ruminantium*, AF401092; *N. helminthoeca*, AF403011; *N. risticii*, AF401089; *N. sennetsu,* AF401088; *R. montanensis,* AY124720; *R. prowazekii,* AF034531; *R. rickettsii,* AF440729; and *W. pipentis,* AF401090. The GenBank accession numbers of the following *gro*ESL genes were used for the determination of sequences identities and construction of polygenetic trees: *A. phagocytophilum*, AF482760; *A. platys,* AF399916; *E. canis*, ECU96731; *E. chaffiensis,* L10917; *E. coli*, X07850; *E. ewingii,* AF195273; *E. muris,* AF210459; *N. helminthoeca,* AY050315; *N. risticii*, ERU24396; *N. sennetsu*, ESU88092; and the SF Agent, AY050314.

Results

Samples from two dogs (Maringá 1, N40-05, mesenteric lymph node; Maringá 2, N20-04, Peyer's patch) were positive by PCR using *Neorickettsia* spp. *rrs*, *rpo*B, and *gro*ESL primers. Positive corresponding bands were more evident after re-amplification of PCR products (Fig. 3.2. A-C). Sequences were obtained from both dogs for the *Neorickettsia* spp. *rrs* gene. However, sequences were only obtained from the Maringá 1 dog for the *N. helminthoeca rpo*B and *gro*ESL genes. All dogs were negative for PCR reactions when the *glt*A gene primers were used. All negative controls yielded no product.

Phylogenetic analyses of the DNA nucleotide sequences of the *rrs*, *rpo*B, and *gro*ESL genes by the neighbor-joining algorithm method demonstrated the marked similarity between the *N. helminthoeca* Maringá strain sequence(s) and the corresponding *N. helminthoeca* sequences deposited in GenBank. The phylogenetic relationship between the Maringá sequences and other members of the *Anaplasmataceae* is demonstrated (Fig. 3.3).

The DNA nucleotide sequences of both Maringá dogs for the *rrs* gene were 100% identical. When these sequences were compared with that of *N. helminthoeca* a sequence identity of 100% was observed (Table 1). A sequence identity of 99% was observed between the two Maringá dogs' *rrs* sequences with *N. sennetsu, N. risticii,* and the SF Agent. Other organisms with similar DNA nucleotide sequences to that of the Maringá strain for the *rrs* gene were *E. bovis* (96%), *E. muris* (95-96%), and *R. tsutsugamushi* (Table 3.1).

Sequence identities of 100%, 82%, and 81% (Table 3.2) were observed between the Maringá 1 dog and *N. helminthoeca, N. risticii,* and *N. sennetsu* for the *rpo*B genes, respectively. Similarly, there was a 100% sequence identity (Table 3.3) between the Maringá 1 dog and *N. helminthoeca* for the *gro*ESL gene sequences; while similarities of 84%, 80%, and 79% were observed with the Maringá strain and *N. sennetsu,* the SF Agent, and *N. risticii*, respectively. No other relatively close organisms were observed when the sequences of the *rpo*B and *gro*ESL genes for the *N. helminthoeca* Maringá strain were compared with other *Anaplasmataceae* (Table 3.2-3.3).

Discussion

This study has demonstrated that two Brazilian dogs (Maringá 1, N40-05; Maringá 2, N20-04), from the city of Maringá, Paraná, with pathological lesions characteristic of those induced by *N. helminthoeca* (Headley et al 2004) as described in SPD (Cordy and Gorham 1950; Jones et al 1997), were infected with a *Neorickettsia* spp., as demonstrated by PCR reaction (Fig. 3.2). Additionally, DNA sequences obtained from Maringá dog 1 demonstrated

to be 100% similar to those of the *N. helminthoeca rrs*, *rpo*B, and *gro*ESL, sequences deposited in the GenBank (Tables 3.1-3.3), while the *rrs* sequences obtained from Maringá 2 were 100% similar to that of *N. helminthoeca* (Table 3.1). Consequently, these findings have confirmed our previously proposed hypothesis that these dogs were infected by this organism (Headley et al., 2004). The organism identified in Maringá, PR, Brazil, has since been called *N. helminthoeca* Maringá strain, and is the first description of this organism beyond the known endemic area of SPD in the Pacific Northwest region of North America and Canada (Headley et al., 2006).

The identification of *N. helminthoeca* from the mesenteric lymph node and the Peyer patches of the small intestine correspond to what is currently known of the pathogenesis of SPD. After ingestion of the infected metacercarie by susceptible dogs, *N. helminthoeca* is transferred, by an unknown mechanism, from the infected fluke to resident intestinal monocytes and macrophages, and subsequently via the circulatory and lymphatic systems, arrives and lodges within somatic and visceral lymph nodes, where primary bacterial multiplication occurs (Rikihisa et al., 2005). This accumulation and proliferation of bacteria produces the marked lymphadenopathy and damage to lymphoid organs that is characteristic of SPD, and observed in these dogs.

In these cases, the actual method of infection-transmission has not been entirely elucidated, and is being investigated. These dogs were maintained on commercially prepared ration that had fish meal as part of its composition, and did not otherwise receive fish as part of their diet. This therefore drastically excludes the conventional method of transmission-infection of *N. helminthoeca*. Additionally, feeding of crude fish to dogs is not a common practice in Brazil. The extrusion process during formulation of this ration is not entirely known. However, during most extrusion processes temperature is elevated to 120150 ^{o}C for about 7-15 seconds. Therefore, it must be determined if this temperature is capable of inactivating the pathogen. *N. helminthoeca* is resistant to lyophilization (Timoney et al., 1992), and remains viable at 4.5 ^{o}C for 48 hours and at 52.5 ^{o}C for 2 minutes (Gorham & Foreyt, 1998). Further, it has been previously demonstrated that smoked fish does not inactivate the helminth-borne pathogen (Farrell et al., 1974), and although only in exceptional cases, *N. helminthoeca* can be transmitted by a non-endoparasitic pathway resulting in SPD in dogs (Bosman et al., 1970). Thus, we are currently investigating the possibility of infection in this case occurring due to ingestion of contaminated-metacercariae within the administered ration.

Due to the difficulty in recovering DNA from these tissue samples and the need to use a highly efficient PCR targeting small DNA regions, the sequences obtained for *N. helminthoeca* Maringá 1 were relatively short (112 bp *rrs*; 143 bp *rpo*B; 92 bp *gro*ESL) when compared to the known sequences deposited in GenBank (*rrs,* 1453 bp; *rpo*B, 464; *gro*ESL*,* 1914 bp). Therefore, more work must be done to obtain comparatively larger sequences for more accurate genotypic comparisons. Furthermore, bacterial isolation and reproduction of SPD in susceptible dogs will be initiated so that better comparative biological analyses can be achieved. Additionally, although all 10 dogs used during this study demonstrated pathological lesions characteristic of SPD, positive PCR results were only obtained from two dogs. This could be related to inadequate quantity or absence of bacterial DNA in the collected samples, and also due to the difficulty while recovering DNA.

This study confirms that *Neorickettsia rrs*, *rpo*B, and *gro*ESL genes (Pretzman et al., 1995; Dumler 2001; Tillardat-Bisch et al., 2003; Rikihisa et al., 2004) are efficient tools for the characterization and phylogenetic analysis of emerging neorickettsial disease in mammals. However, in this study the *rpo*B and *gro*ESL genes have demonstrated marked phylogenetic variability within the *Anaplasmataceae* family when compared with the *rrs* gene (Fig. 3.3), resulting in better phylogenetic analysis; similar results have also been reported (Dumler 2001; Tillardat-Bisch et al 2003; Rikihisa et al 2004). This is because although the *rrs* gene is well conserved within *Neorickettsia* spp., some of these areas are homologous with other members of the *Anaplasmataceae* family, resulting in closer similarity with other organisms (Table 3.1; Fig. 3.3). Therefore, characterization of neorickettsial agents requires the utilization of the *rrs* gene in association with the *rpo*B and/or *gro*ESL genes.

During this study and other previous experiences at our laboratory, we were unable to obtain adequate results with the citrate synthase genes (Inokuma et al., 2001) for the identification of *N. helminthoeca* by PCR, so the utilization of this gene for the characterization of neorickettsial diseases should be reviewed. We therefore suggest that the *Neorickettsia* spp. *rrs* primers (NeoSH-F and NeoSHR) be used as preliminary molecular indicators for the presence of neorickettsial disease in mammalian tissues, due to the marked degree of similarity (99-100%) demonstrated between *N. helminthoeca*, *N. helminthoeca* Maringá strain, *N. sennetsu*, *N. risticii*, and the SF Agent during this study (Table 1).

The distribution of this disease in the Brazilian dog population is unknown. Canine parvovirus, in which the clinical intestinal manifestations are often indistinguishable from those seen in SPD (Gorham and Foreyt, 1998), is one of the principal causes of death in some urban dog populations in Brazil. Therefore, in these dog populations, SPD should be

considered as a differential diagnosis in suspected cases of canine parvovirus, principally those accompanied with generalized lymphadenopathy.

The biology of neorickettsial agents has been unfolded in recent years, where highly complex life cycles that involves mature and immature helminths, aquatic insects, several different mammals, including even bats, have been incriminated (Chae, 2000; Pusterla et al 2000; Pusterla, 2003; Gibson, 2005). While SPD is caused by *Neorickettsia helminthoeca*, infections by other *Neorickettsia* spp., (*N. risticii,* Potomac horse fever; *N. sennetsu,* sennetsu fever; and the SF Agent, "Hyuganetsu" disease), illustrate the broad potential of these widely distributed species to infect and cause disease in the mammalian and human host. Additionally, newly discovered neorickettsial and rickettsial organisms results in life-threatening emerging infectious diseases (Walker & Dumler, 1996), some in geographical regions previously considered as non-endemic. Consequently, the detection of *N. helminthoeca* in dogs from Brazil further extends the range of this species and warrants a broad search for infections and spectrum of disease caused by neorickettsial agents in animals and man. Further, neorickettsial pathogens can coexist within their trematode hosts, are maintained unmodified throughout the biological cycle of the trematode and are readily transferred to mammalian monocytes.

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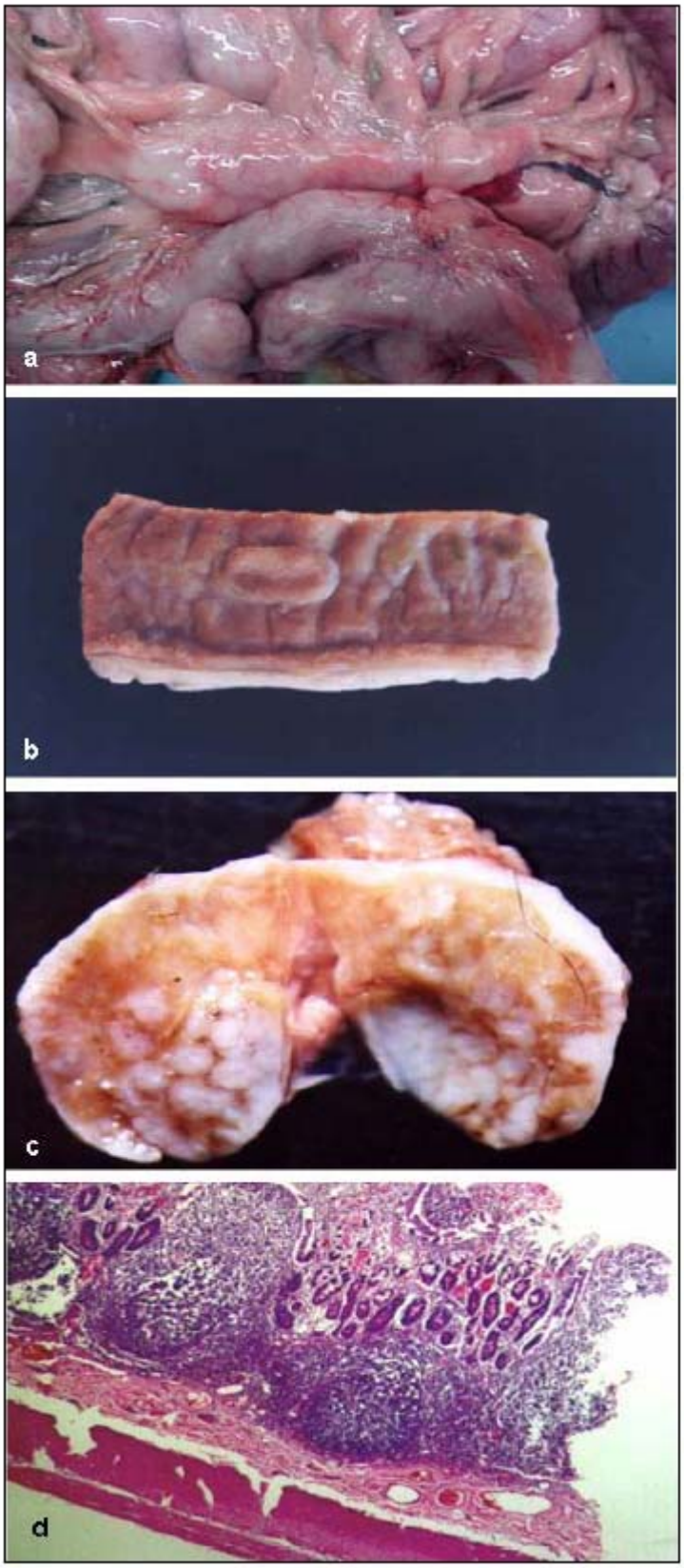

Figure 3.1. Salmon poisoning disease of dogs. Observe severe hypertrophy of mesenteric lymph node (a), and of intestinal Peyer's patch and lymphoid tissue (b). Cross section of mesenteric lymph node; there is loss of medullar-cortical differentiation and white randomly distributed white follicles principally at the cortical zone (c). Photomicrograph; observe severe proliferation of intestinal lymphoid tissue and hemorrhage. HE, Obj. 10 x. (d).

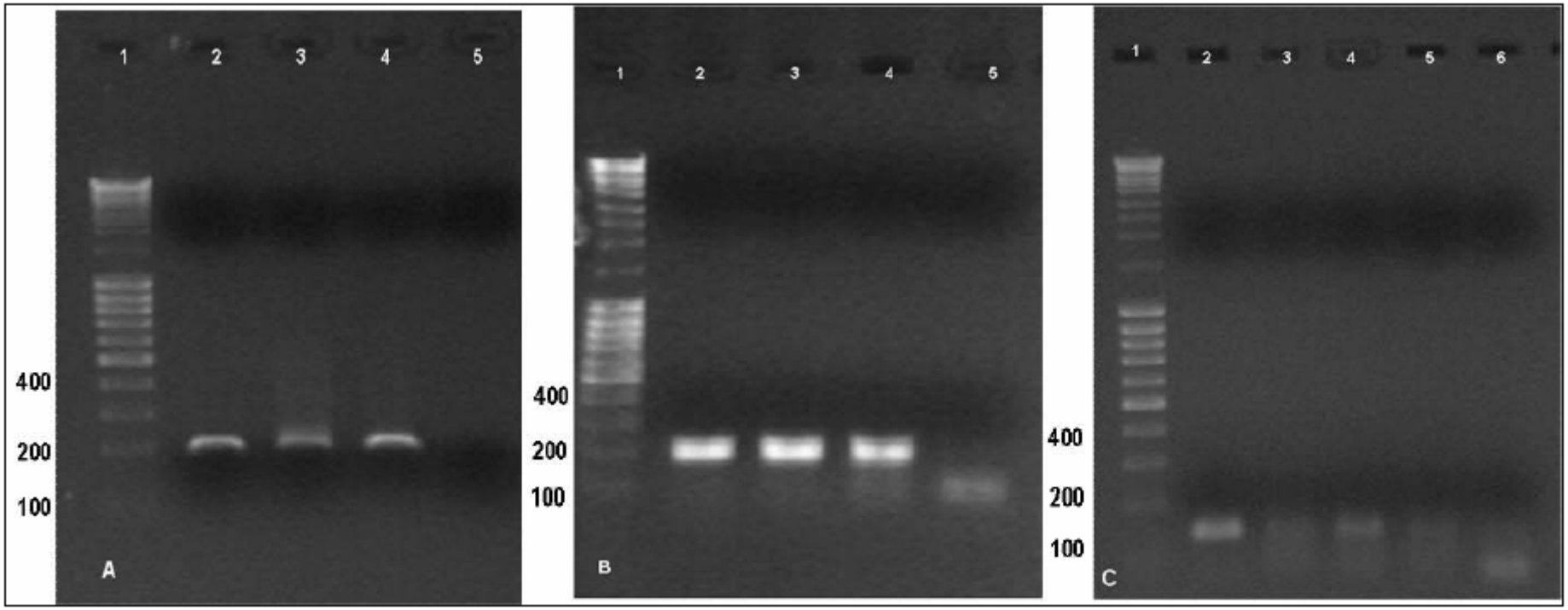

Figure 3.2. PCR results of the re-amplified products of the two Maringá dogs with primer sets NeoSH-F - NeoSH-R, 207 bp (A), NH-rpoB-F – NH-rpoB-R, 196 bp (B); NH-*gro*ESL-F - NH-*gro*ESL-R, 141 bp (C). Lanes: 1, DNA Ladder (Fermentas, Hanover, MD, USA); 2, *N. helminthoeca,* positive control; 3, Dog N20/04, Peyer's patch, small intestine; 4, Dog N40/05, mesenteric lymph node; and 5, DNA Nuclease-free water (Promega Corporation, Madison, WI, USA); Photos A and B. Lanes: 1, DNA Ladder; 2, *N. helminthoeca*, positive control; 3, Dog N20/04, Peyer's patch, small intestine; 4, Dog N40/05, mesenteric lymph node; and 5, Dog N20/04, cecum; and 6, DNA Nuclease-free water; Photo C. The sizes of the corresponding PCR fragments are indicated in base pairs.

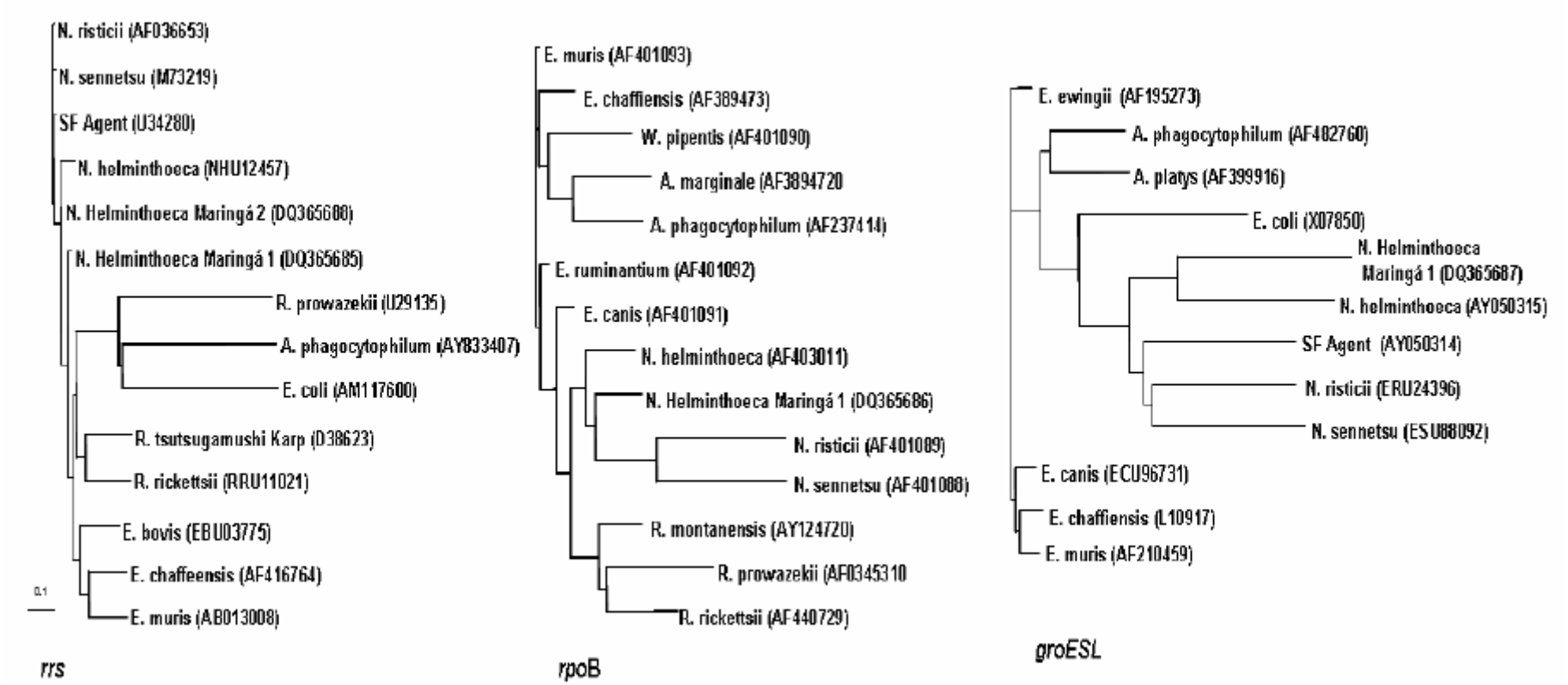

Figure 3.3. Phylogenetic trees based on the *rrs*, *rpo*B, and *gro*ESL gene sequences of *Anaplasmataceae* families. Trees were constructed by the Vector NTI Advance™ 10 Software (bars represent substitutions per 1,000 bp; GenBank accession numbers are given in parentheses).

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Table 3.1. Nucleotide sequence identities of *Anaplasmataceae* for the 16S ribosomal RNA (*rrs*) gene.

Organism	% Nucleotide sequence identity[a]												
	E. coli	*E. chaffeensis*	*E. muris*	*N. helminthoeca* Maringá 2	*N. helminthoeca* Maringá 1	*N. helminthoeca*	SF Agent	*N. risticii*	*N. sennetsu*	*E. bovis*	*R. tsutsugamushi* Karp	*R. prowazekii*	*R. rickettsii*
A. phagocytophilum	36	43	43	52	51	44	43	43	43	43	40	34	41
E. coli		42	42	53	51	42	43	42	42	42	40	33	43
E. chaffeensis			98	95	96	86	84	84	84	91	84	46	83
E. muris				95	96	85	84	84	84	91	84	46	83
N. helminthoeca Maringá 2 [b]					100	100	99	99	99	96	92	48	93
N. helminthoeca Maringá 1 [c]						100	99	99	99	96	93	49	94
N helminthoeca							95	96	95	84	82	43	82
SF Agent								99	99	84	82	42	82
N. risticii									99	84	81	42	82
N. sennetsu										84	81	43	82
E. bovis											83	44	83
R. tsutsugamushi Karp												45	90
R. prowazekii													43

[a] The values represent the percent of nucleotide sequence identity, and were determined by the Vector NTI Advance™10 Software.
[b] *N. helminthoeca* Maringá 2, partial sequences 107 bp.
[c] *N. helminthoeca* Maringá 1, partial sequences, 112 bp.

Table 3.2. Nucleotide sequence identities of *Anaplasmataceae* for the RNA polymerase β-subunit (*rpo*B) gene.

Organism	% Nucleotide sequence identity[a]												
	A. phagocytophilum	*W. pipentis*	*E. chaffiensis*	*E. canis*	*E. muris*	*N. risticii*	*N. sennetsu*	*N. helminthoeca* Maringá 1	*N. helminthoeca*	*R. prowazekii*	*R. rickettsii*	*R. montanensis*	*E. ruminantium*
A. marginale	76	57	69	73	73	46	46	57	57	56	61	54	73
A. phagocytophilum		60	71	75	74	51	51	64	56	58	60	56	76
W. pipentis			63	73	74	46	46	34	40	56	63	58	74
E. chaffiensis				90	88	50	49	62	56	61	66	58	87
E. canis					88	57	57	0	0	68	65	0	88
E. muris						62	63	0	0	68	64	0	95
N. risticii							94	82	76	51	61	47	56
N. sennetsu								81	74	50	61	47	56
N. helminthoeca Maringá 1 [b]									100	59	0	0	0
N. helminthoeca										54	0	0	0
R. prowazekii											88	95	67
R. rickettsii												0	63
R. montanensis													0

[a] The values represent the percent of nucleotide sequence identity, and were determined by the Vector NTI Advance™10 Software.

[b] *N. helminthoeca* Maringá 1, partial sequences, 143 bp.

Table 3.3. Nucleotide sequence identities of *Anaplasmataceae* for the heat shock protein (*gro*ESL) gene.

Organism	% Nucleotide sequence identity[a]										
	A. platys	*N. helminthoeca* Maringá 1	*N. helminthoeca*	*N. risticii*	*N sennetsu*	SF Agent	*E. coli*	*E. canis*	*E. chaffiensis*	*E. muris*	*E. ewingii*
A. phagocytophilum	81	47	57	59	57	58	52	76	75	76	75
A. platys		49	57	57	56	57	54	74	73	75	74
N. helminthoeca Maringá 1[b]			100	79	84	80	56	47	48	47	46
N. helminthoeca				79	78	79	50	58	55	57	57
N. risticii					96	92	55	60	59	58	59
N. sennetsu						91	48	58	52	56	57
SF Agent							48	59	54	58	58
E. coli								53	47	53	53
E. canis									92	92	89
E. chaffiensis										94	90
E. muris											90

[a] The values represent the percent of nucleotide sequence identity, and were determined by the Vector NTI Advance™10 Software.
[b] *N. helminthoeca* Maringá 1, partial sequences, 92 bp.

Conclusions

1. Two dogs from the city of Maringá, PR, with gross and histopathological lesions consistent to those described in salmon poisoning disease (SPD) were positive for *Neorickettsia helminthoeca* by PCR using primers targeted at the *rrs*, *rpo*B, and *gro*ESL genes.
2. The partial DNA nucleotide sequences, based on the *rrs* (112 bp), *rpo*B (143 bp), and *gro*ESL (92 bp) genes, of one of these dogs (Maringá 1) was 100% similar to that of *N. helminthoeca*.
3. This is the first description of *N. helminthoeca* and SPD beyond the known endemic geographic location of the Pacific Northwest of the United States of America, and Canada.

4 CONCLUSÕES

1. Dois cães (N 40-05, Maringá 1; N 20-04, Maringá 2) no Brasil com alterações macroscópicas e histológicas consistentes com aquelas descritas na intoxicação por salmão (SPD) foram positivos para a *Neorickettsia helminthoeca* pela PCR a partir de primers baseados nos genes da 16S ribossomal RNA (*rrs*), β-subunidade *da RNA* polimerase (*rpo*B) e da proteína de choque-térmico (*gro*ESL).
2. Os dois cães apresentaram seqüências com 100% de identidade para o gênero *Neorickettsia*.
3. As seqüências nucleotídeas parciais de amostras de um cão (Maringá 1) demonstraram identidade de 100% para os genes de *rrs* (112 bp), *rpo*B (143 bp) e *gro*ESL (92 bp) da *N. helminthoeca*.
4. As alterações macroscópicas da SPD no Brasil foram caracterizadas por linfadenopatia, hipertrofia das placas de Peyer, hipertrofia do tecido linfóide intestinal e enterite hemorrágica.
5. As alterações histológicas da SPD no Brasil foram caracterizadas principalmente por hiperplasia dos tecidos linfóides e das células reticuloendoteliais associadas aos organismos intralesionais e intracitoplasmáticos consistentes com a *N. helminthoeca*.

5 PERSPECTIVAS

The first diagnosis of *Neorickettsia helminthoeca* and salmon poisoning disease (SPD), beyond the known endemic region of the disease, in Brazilian dogs is a great achievement for the scientific community of the State of Paraná, but more specifically, for all engaged in research at the Universidade Estadual de Londrina. Additionally, this diagnosis has thus resulted in a new line of research in Brazil that would have to be aimed at resolving unanswered questions raised during this research. These doubts include information relative to the epidemiology, pathogenesis, and life cycle of organisms associated with *N. helminthoeca* in Brazil.

In association with colleagues at the Johns Hopkins University, antibodies (with relatively high titles) have already been developed in immunized rabbits against *N. helminthoeca*. This would soon be used for the realization of immunohistochemical studies to visualize the distribution of bacterial antigens in formalin-fixed paraffin-embedded tissues of the dogs studied during this thesis, and would form the base for the immunohistochemical diagnosis of SPD in Brazil. Additionally, antigens slides of *N. helminthoeca* for the realization of Indirect Immunofluorescent Antibody Tests have been prepared (at Johns Hopkins University) and would be an efficient supportive tool for the quick diagnosis of SPD in susceptible dog populations.

In Brazil, canine parvovirus (CPV) induced hemorrhagic enteritis is frequently diagnosed based primarily on the clinical manifestations and/or the histological lesions associated with the disease. With the confirmation of SPD in Brazil, immunohistochemical retrospective studies using formalin-fixed paraffin-embedded tissues should be realized to determine if all previously diagnosed cases of CPV were as diagnosed, since clinically the hemorrhagic enteritis of these two diseases cannot be differentiated. Additionally, dogs with hemorrhagic enteritis associated with lymphadenopathy should be regarded as suspects of SPD.

During this study the clinical manifestations of SPD were not observed in these dogs. Therefore there is an urgent need to reproduce this disease in susceptible dogs, isolate the bacteria, and obtain correspondingly larger DNA sequences so that additional phylogenetic analyses could be realized, and Koch's Postulates can be filled.

It is proposed that integrated epidemiological studies of SPD in Brazil be initiated and should be focused on a combination of or one of the following:

1. Collection of whole blood from dogs attended at veterinary clinics with clinical manifestations of hemorrhagic enteritis associated with lymphadenopathy for PCR/serological screening;
2. Search and collection of pleurocerid snails and digenean trematodes, beginning in the city of Maringá, Paraná, in an attempt to define the biological cycle of the organisms associated with *N. helminthoeca* in Brazil;
1. Utilize molecular biology techniques to investigate the possibility of infection-transmission in Brazilian dogs by *N. helminthoeca* occurring due to infected metacercarie in contaminated commercially produced canine ration;
2. Evaluate the potential risk to public health of infections by neorickettsial organisms in humans, principally in communities where the ingestion of crude fish is common.

The pathogenesis of *N. helminthoeca* is not totally elucidated; it is not known how the bacterium leaves the intestinal trematode and enters the mammalian host monocytes. Therefore, the host-pathogen relationship of *N. helminthoeca* must be studied, either by the use of mouse models or “in vitro” studies using DH 82 cells. Additionally, all dogs used during this experiment were sacrificed for other research purposes; there were no acute deaths as described in SPD of the Pacific Northwest of the USA or in

Canada. Therefore immunological/microbiological studies must be initiated to determine if the *N. helminthoeca* Maringá strain is less virulent than the bacteria described in the USA and Canada.

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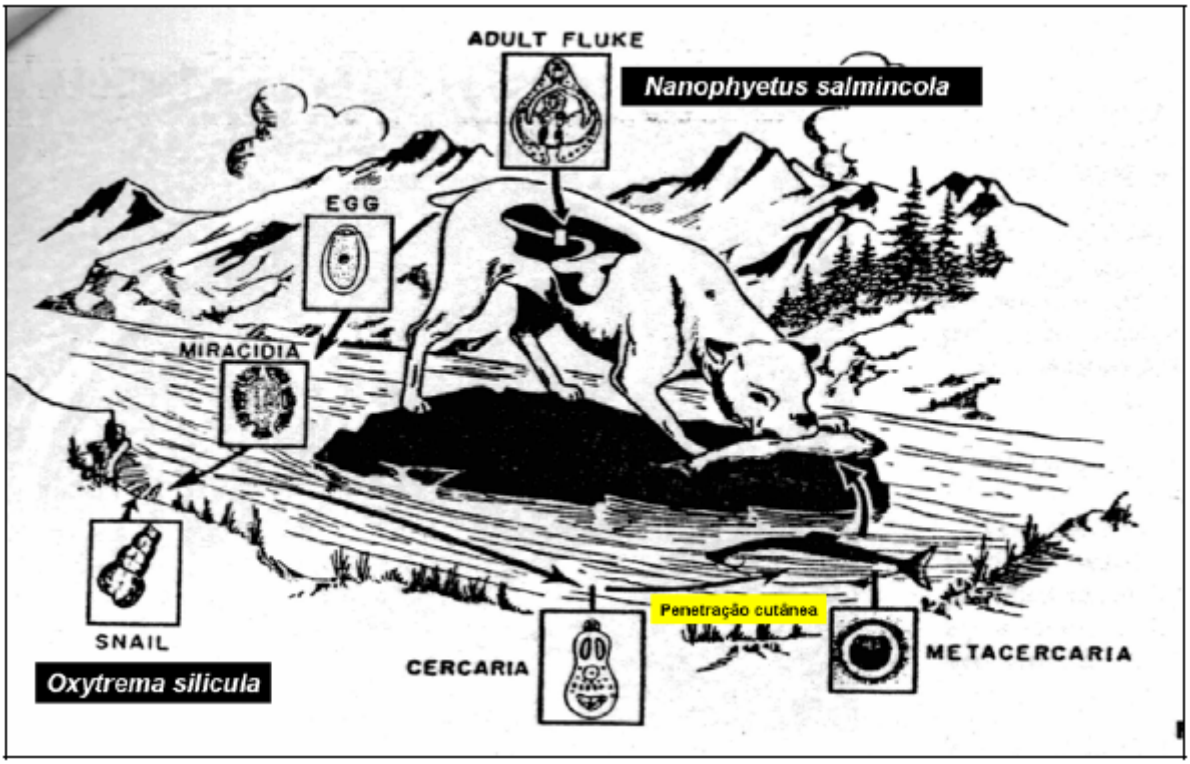
APÊNDICES

Apêndice 1 - Endemic region of Salmon Poisoning Disease

This area (▮) is delimited by the Sacramento River in northwest California, passing through the western coast of Oregon, to the Olympic Peninsula of southwestern Washington, and extending inland to the Pacific slopes of the Cascade Mountains.

Apêndice 2 - Life cycle of *Nanophyetus salmincola*[1]



[1] Adapted from Gorham J. R.; W. J. Foreyt**.** Salmon poisoning disease. In: **Infectious diseases of the dog and cat**, C. E. Green, (Ed), 2nd Ed., Philadelphia: WB Saunders. p. 35-139,1998.

Apêndice 3 - PCR Touch-down Protocol

Initial denaturation at 94°C for 2 min.

Denaturation at 94°C for 30 sec.
Annealing at 67°C for 30 sec.
Extension at 72°C for 30 sec.
for 2 cycles

Denaturation at 94°C for 30 sec.
Annealing at 64°C for 30 sec.
Extension at 72°C for 30 sec.
for 2 cycles

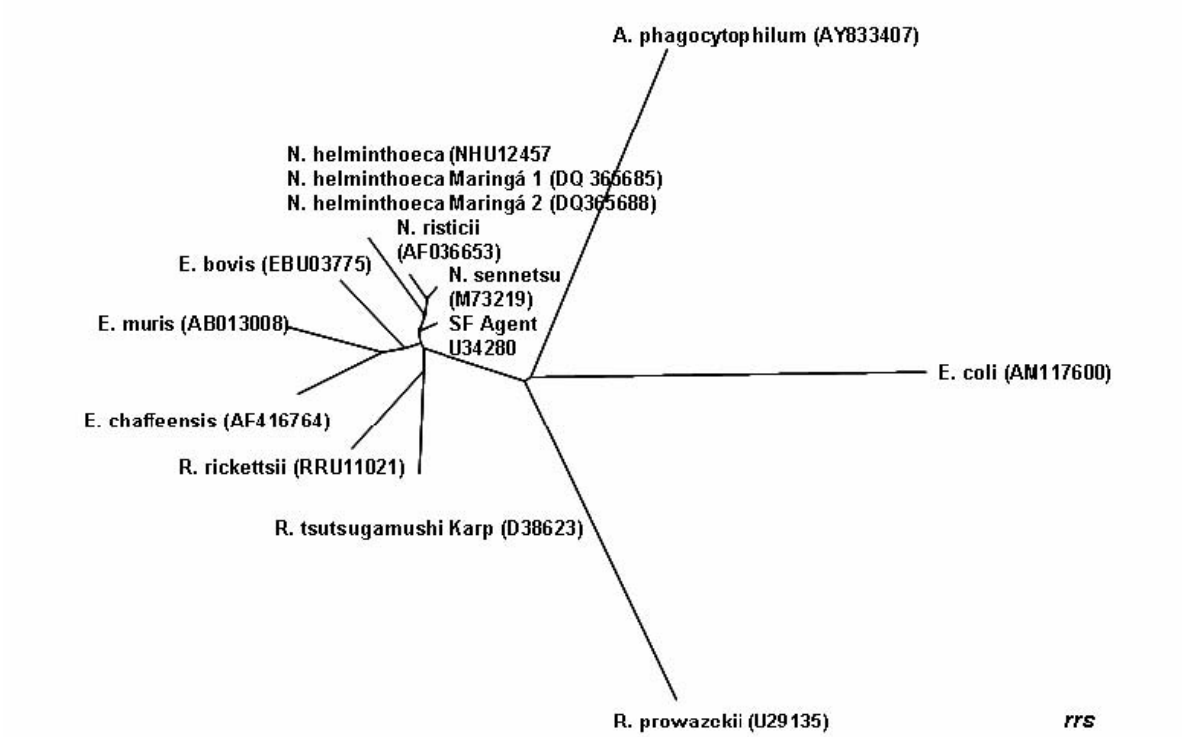
Denaturation at 94°C for 30 sec.
Annealing at 61°C for 30 sec.
Extension at 72°C for 30 sec.
for 2 cycles

Denaturation at 94°C for 30 sec.
Annealing at 58°C for 30 sec.
Extension at 72°C for 30 sec.
for 2 cycles

Denaturation at 94°C for 30 sec.
Annealing at 55°C for 30 sec.
Extension at 72°C for 30 sec.
for 28 cycles

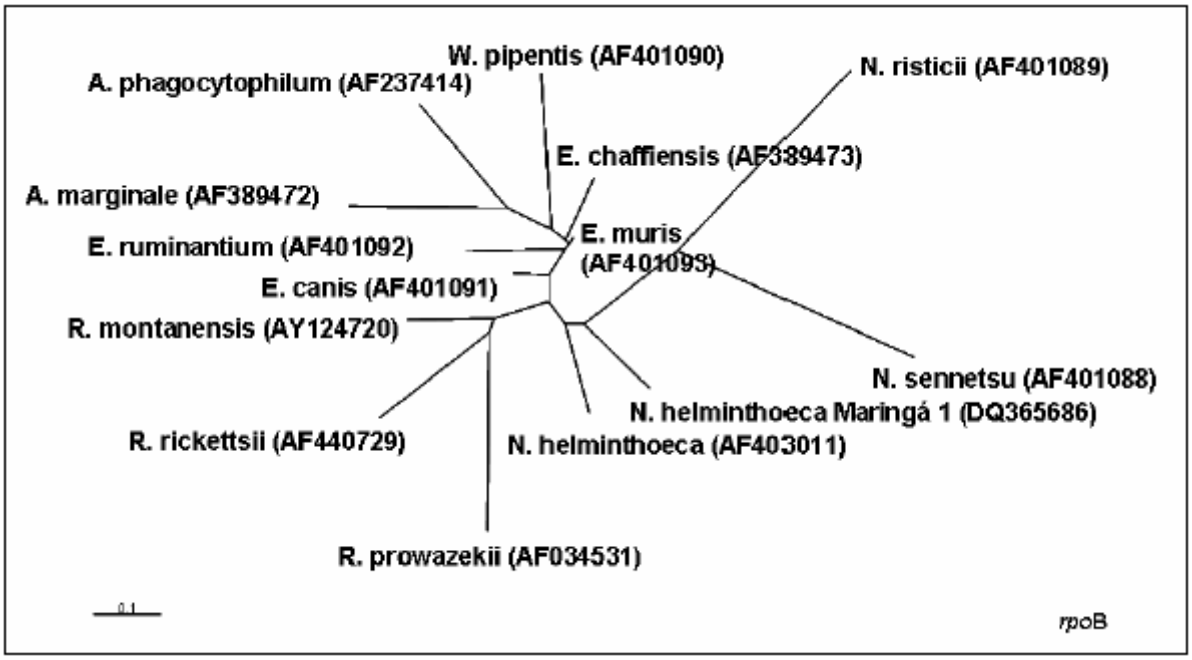
Final extension at 72°C for 5 min.

Apêndice 4 – Phylogentic relationship of *Anaplasmataceae* for the *rrs* gene[1].



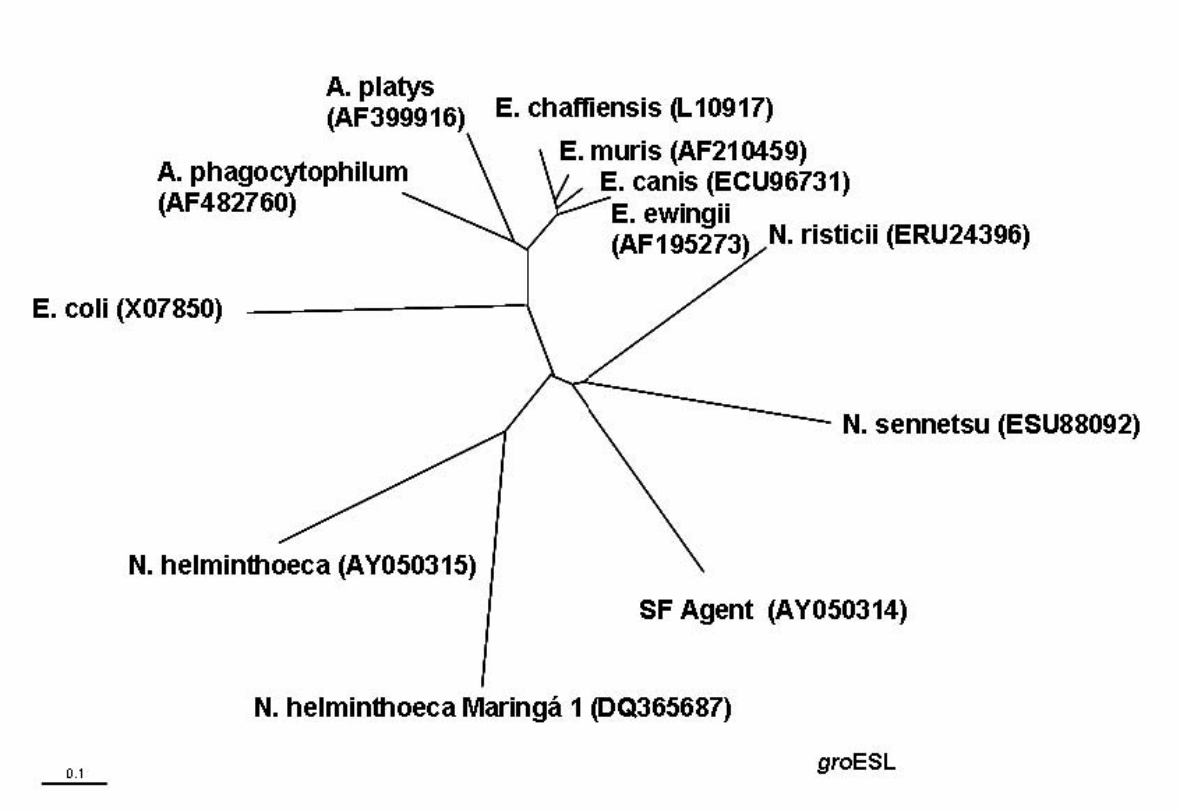
[1] Tree was constructed by the Vector NTI Advance™10 Software (bars represent substitutions per 1,000 bp; GenBank sequence accession numbers are given in parenthesis).

Apêndice 5 – Phylogentic relationship of *Anaplasmataceae* for the *rop*B gene[1].



[1] Tree was constructed by the Vector NTI Advance™ 10 Software (bars represent substitutions per 1,000 bp; GenBank sequence accession numbers are given in parenthesis).

Apêndice 6 – Phylogentic relationship of *Anaplasmataceae* for the *gro*ESL gene[1].



[1] Tree was constructed by the Vector NTI Advance™10 Software (bars represent substitutions per 1,000 bp; GenBank sequence accession numbers are given in parenthesis).