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Nanophyetus salmincola, vector of the salmon poisoning disease agent *Neorickettsia helminthoeca*, harbors a second pathogenic *Neorickettsia* species

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Abstract

The trematode *Nanophyetus salmincola* is known as the carrier of *Neorickettsia helminthoeca*, an obligate intracellular endosymbiotic bacterium that causes salmon poisoning disease (SPD), a fatal disease of dogs. The bacteria are maintained through the complex life cycle of *N. salmincola* that involves snails *Juga plicifera* as the first intermediate host, salmonid fishes as the second intermediate host and fish-eating mammals as definitive hosts. *N. salmincola* was also found to harbor a second species of *Neorickettsia* that causes the Elokomin fluke fever disease (EFF) which has clinical signs similar to SPD in bears, but only low grade illness in dogs. The EFF agent has not been sequenced. In this study we identified *N. salmincola* as the vector of yet additional species of *Neorickettsia* known as *Stellanchasmus falcatu* (SF) agent using DNA sequencing.

Keywords

Neorickettsia; salmon poisoning of dogs; SF agent; EFF agent; molecular diagnostics

1.) Introduction

Research focusing on salmon poisoning disease (SPD) in dogs began in 1911, when Pernot (1911) demonstrated that SPD was caused by an infectious agent. In early 1930's Simms et al. (1932) demonstrated conclusively that metacercariae and adult flukes of *Nanophyetus salmincola* caused SPD when fed or injected into dogs. They suspected that the infection

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was of rickettsial or haemosporidian origin. Their hypothesis was confirmed when Cordy and Gorham (1950) described intracytoplasmic rickettsial-like organisms in reticuloendothelial cells from Giemsa-stained lymph node impression smears taken from a dog that died of salmon poisoning. Philip et al. (1953) named the agent *Neorickettsia helminthoeca*.

In nature, dogs acquire infection by consuming fish containing *N. salmincola*-infected metacercariae. When dogs eat infected fish, the ingested metacercariae mature to adult flukes in the small intestine. Adult flukes begin producing eggs in 5–10 days, which corresponds closely to the normal pre-patent period of SPD symptoms in dogs. If left untreated, most dogs die within 6 to 10 days after onset of clinical symptoms. During the rapid course of disease, gross pathology includes enlarged and fleshy visceral lymph nodes, often with areas of hemorrhage and necrosis, marked splenomegaly with prominent white follicles (Timoney et al., 1992; Jones et al.,1997). Frequent severe hemorrhaging of the intestinal tracts with a mucoid appearance, intussusception of the small intestine, hepatic rupture with peritonitis, and hemorrhage in both the gall and urinary bladders are also observed (Jones et al., 1997).

In 1973, *N. salmincola* was identified to harbor a second species of *Neorickettsia* (Farrell et al., 1973). Farrell and colleagues were trying to determine if black bears, *Ursus americanus*, could act as natural reservoirs of SPD. Metacercarial-infected trout from Elokomin river were fed to 6 captive bears, 4 of which developed fever, anorexia and ‘lassitude’. Upon necropsy, the sick bears had swollen mesenteric lymph nodes suggestive of salmon poisoning. Three of the bears had rickettsial bodies in lymph node impression smears. Following injection of individual lymph suspensions from 5 necropsied bears into groups of 4 to 6 dogs each, 72% of the dogs developed low-grade fever and diarrhea for 4 to 12 days, but did not die. Farrell et al. (1973) named the disease “Elokomin fluke fever” (EFF). Subsequent studies found that EFF agent was immunologically distinguishable from *N. helminthoeca* (Kitao et al., 1973; Sakawa et al., 1973). However, DNA from EFF agent was never sequenced, making it difficult to identify the species of *Neorickettsia* responsible for the disease.

2.) Materials and Methods

In this study, we used real-time PCR based detection to screen *N. salmincola* metacercariae found in kidneys of several adult Chinook salmon from the Willamette River, Oregon for neorickettsial DNA. These adult fish return to fresh water in the early summer and spawn in the fall. They exhibit > 90% prevalence of infection with *N. salmincola* and intensities of infection that reach well over 1,000 metacercariae/gram kidney as the summer progresses (Kent et al. 2013). Likewise, heavy infections also occur in other organs (Fig. 1a, b) and the somatic muscle. A total of 334 metacercariae of *N. salmincola* were screened for the presence of *Neorickettsia*. Metacercariae were removed from the kidneys using fine needles under a stereomicroscope, rinsed in distilled water and genomic DNA was extracted according to Tkach and Pawlowski (1999) or using the Genomic DNA-Tissue Miniprep kit (Zymo Research, Irvine, CA). DNA extracts were first tested for the presence of *Neorickettsia* following a real-time PCR protocol targeting a 152bp portion of the 3' end of

the heat shock protein coding gene, GroEL, described by Greiman et al. (2014). Samples that tested positive for neorickettsial DNA were verified using a nested PCR protocol amplifying a 1370 bp fragment of the 16S rRNA gene as described by Greiman et al. (2014) with subsequent sequencing of PCR products. Identification of *Neorickettsia* positive metacercariae was confirmed by partial sequences of the nuclear ribosomal 28S gene. Additionally, two blood samples from dogs in Oregon, previously treated for SPD and recovered, but exhibiting symptoms many months after treatment, were screened for *Neorickettsia*.. Sequences from this study were submitted to GenBank (SF agent KX462530; *Neorickettsia helminthoeca* KX462531: *Nanophyetus salmincola* KX462529).

3.) Results

As a result, we found that *N. salmincola* carries yet another species of *Neorickettsia*, known as *Stellanchasmus falcatus* (SF) agent. We have isolated 16S DNA of SF agent from metacercariae of *N. salmincola* found in kidneys of 3 different individuals of Chinook salmon (334 metacercariae screened) in Oregon. The 16S sequence of SF agent found in our study from metacercariae of *N. salmincola* matches 100% the sequence of SF agent from Japan found in metacercariae of *S. falcatus* (GenBank U34280). This is the first report of this bacterium from *Nanophyetus* and from a salmonid fish (family Salmonidae) anywhere in the world.

4.) Discussion

SF agent was initially discovered in metacercariae of digeneans *Stellantchasmus falcatus* (family Heterophyidae) infesting grey mullet in Japan (Fukuda et al., 1973) and was also recently found in another heterophyid, *Metagonimoides oregonensis*, in Florida (Greiman et al., 2014). SF agent is known to cause mild fever in dogs and can be isolated from the blood of infected dogs (Fukuda and Yamamoto, 1981).

Finding of SF agent in *N. salmincola* is interesting for several reasons. It is the first report of SF agent in a non-heterophyid digenean, leading to the possibility that SF agent could be found in a greater diversity of digeneans than other genotypes of *Neorickettsia*. Second, this is only the second report of a single digenean species serving as the host for more than one *Neorickettsia* species; the first such record was *N. salmincola* harboring EFF (Farrell et al., 1973). This demonstrates the possibility of host-switching among species of *Neorickettsia*, especially considering the high host specificity of *Neoricekttsia* (Tkach et al., 2012, Greiman et al., 2014; Greiman et al., in press). The most interesting, however, is the potential role of different *Neorickettsia* species as agents of dog diseases. As mentioned above, *N. salmincola* is the host of *N. helminthoeca*, the agent of SPD. It is also well known that dogs infected with *N. helminthoeca* become immune to re-infection upon recovery (Bosman et al., 1970; Vaughan et al., 2012). However, one of us (MK) gave a lecture on salmon poisoning to at a local veterinary symposium (Marion Polk Veterinary Association CE meeting, 16–17 November, 2013). Of the some 40 veterinarians at the lecture, many responded that they have seen repeat cases of salmon poisoning in local dogs, but usually in a milder form.

Sykes et al. (2010) also reported a recurrent infection. SF agent is known to cause mild clinical symptoms in experimentally infected dogs, therefore, it could be possible that dogs in Oregon may become infected both with *N. helminthoeca* and SF agent. This hypothesis seems to be feasible considering that the two forms of *Neorickettsia* can use the same digenean host, *N. salmincola*, and there is weak antigenic cross-reactivity between SF agent and *N. helminthoeca* (Rikihisa et al., 2004). As stated above, *N. salmincola* is also known as the host of EFF agent that causes severe illness in bears, but produces only low-grade fever and diarrhea for 4 to 12 days in dogs. Based on the similarities in disease symptoms in dogs caused by the EFF agent (that was never sequenced) and SF agent, we hypothesize that SF agent may be the cause of EFF. It is also important to note that SF agent has been isolated from rainbow trout tissue in Oregon, but not from digeneans (Rikihisa et al., 2004). It is possible that the source of SF agent in the tissue of rainbow trout were metacercariae of *N. salmincola*.

Surprisingly, in this study we did not isolate DNA of *N. helminthoeca* from metacercarie of *N. samincola* obtained from Chinook salmon kidneys. However, we found *N. helminthoeca* in blood samples taken from two sick dogs brought into a veterinary clinic in the Willamette Valley in Oregon. These dogs were previously treated for symptoms of SPD and returned to the clinic showing new symptoms of the disease. Based on our results, it is possible that the dogs were previously infected with a species of *Neorickettsia* other than *N. helminthoeca*, maybe SF/EFF agent, and were secondarily infected with *N. helminthoeca*. Alternatively, they simply may not have had immunological protection after recovering from their first infection with *N. helminthoeca*, but we have no evidence of this at this time. Further screening of blood/tissue from sick dogs in the Pacific Northwest, especially dogs previously treated for SPD that develop symptoms of the disease second time, is needed to determine whether SF agent is the cause of subsequent infections.

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Highlights

- *Neorickettsia* are intracellular bacteria that cause diseases in domestic animals and humans
- *Nanophyetus* flukes were known as hosts of two neorickettsial pathogens
- Our study has found a third *Neorickettsia* in *Nanophyetus*
- This discovery may explain secondary infections of dogs that are supposed to be immune
- We hypothesize that Elokomin Fluke Fever agent and SF agent are the same *Neorickettsia*

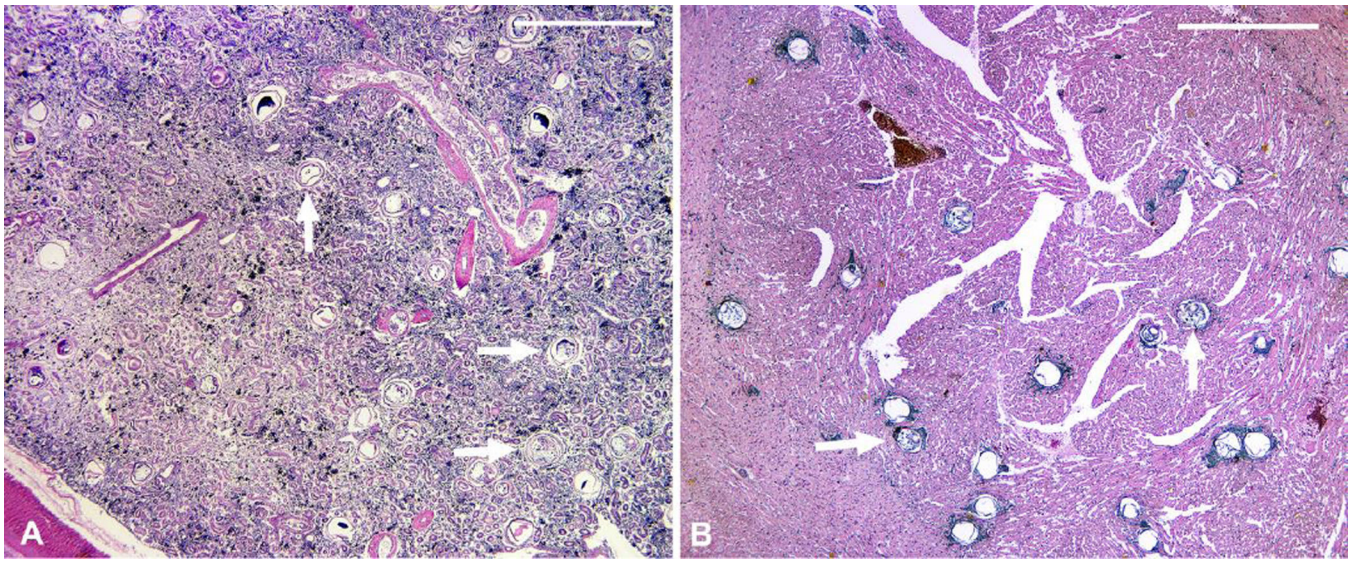

Figure 1.

Metacercariae of *Nanophyetus salmincola* in histological section of (A) kidney of Chinook salmon, and (B) heart tissue of Chinook salmon. Metacercariae on both images are indicated by arrows. Scale bar = 1 mm.