MORPHOLOGICAL AND MOLECULAR ANALYSIS OF ASCARIDOIDEA FROM THE NORTHERN HOLARCTIC

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This dissertation is based on a set of publications presenting the results of studies on Ascaridoidea (Nematoda: Chromadorea), in the system of De Ley and Blaxter 2002, vertebrate endoparasites of major veterinary, medical and economic importance. This superfamily includes about 50 genera, whose representatives are widely distributed in their intermediate, paratenic and final hosts in both hemispheres. Because of their importance as pathogens, relatively large size and common distribution, representatives of Ascaridoidea are among the most widely studied nematodes parasitizing in vertebrates. The Ascaridoidea structural features, particularly morphological ones, exhibit considerable variation in the genera and in some cases, species. Likewise, their life cycles vary. Like most parasites, nematodes from Ascaridoidea superfamily shows a specificity for host. In the waters of the North Holarctic from Ascaridoidea significant are families Anisakidae and Raphidascarididae, which are listed numerously. They characterize complex life cycles involving multiple species of cephalopods, fish, fish-eating birds and marine mammals.

Anisakidae reach sexual maturity in warm-blooded animals (marine mammals and fish-eating birds), and Raphidascarididae mainly in fish, the most commonly parasitizing in the stomach and intestines. Their life cycle includes five stages (L1–L4 and adult stage) separated by four molts. Fertilized eggs in the feces of the definitive host are released into the water. In the thick cuticle the L2 larvae (or for some species L3) develop, in the marine environment leave the eggs and then can be swallowed by a first intermediate hosts – planktonic and benthic invertebrates. *Pseudoterranova* spp. have a benthic life cycle, because the larvae of species of this genus are not able to swim, as opposed to larvae within the genus *Anisakis* spp. and *Contracaecum* spp. Moulting, if it has not happened before, occurs in the first intermediate host and the larvae attains stage L3, which is invasive for vertebrates. Second intermediate or paratenic hosts are fish (e.g. herring, mackerel, salmon), which become infected by eating crustaceans with larvae. In fish larvae L3 settled mostly on the peritoneal lining of the body cavity and covers the organs (e.g. *A. simplex*), although some show a preference to settle in specific organs (e.g. *P. decipiens*). The larvae of this form are invasive for the final host (species of the genus *Anisakis* for cetaceans, *Pseudoterranova* for pinnipeds, *Contracaecum* for pinnipeds and fish-eating birds, *Hysterothylacium* for fish). When the larva is swallowed by the definitive host, grows and moults to stage L4, and then reaches sexual maturity. In the life cycle often appears paratenic host – predatory fish (e.g. halibuts) and cephalopods, in which bodies large numbers of larvae accumulate, but do not undergo further development.

Anisakidae family includes species of great economic importance. Parasites damage host tissues and weaken it. Affecting the condition of organisms are the cause of diseases, damage in the cultures and economic losses. The big problem is that these nematodes reduce the commercial value of the fish meat. Mass parasites occurrence cause the fish are not suitable for consumption for aesthetic considerations and often also change the technological properties of the raw material. In addition, the removal of parasites from fish is labor intensive and entails additional costs. Moreover, the larvae of species which belong to this family are pathogenic to humans who eat raw or undercooked fish meat, causing zoonoses called anisakidosis. It reveals by fever, abdominal pain, nausea and vomiting. Species from genera: *Anisakis*, *Contracaecum*, *Pseudoterranova*, and less often from family Raphidascarididae – *Hysterothylacium* are causing this disease. By changing dietary preferences (including sushi or sashimi popularity), anisakidosis is more numerous, and due to use a better diagnostic methods, it is identified more often. However, despite this many cases remain undiagnosed, mainly due to non-specific symptoms, such as diarrhea, vomiting, tingling sensation in the throat. In addition, more cases of allergy to the third stage larvae (L3) of *Anisakis* spp. are reported after consumption of properly prepared fish. It is known that allergens of larvae of the genus *Anisakis* are heat-resistant and thermal treatment does not destroy their allergenic ability. The diagnosis of anisakidosis is difficult due to cross-reactions between the antigens of other species of nematodes of the genus *Anisakis*, *Ascaris*, *Hysterothylacium*, *Toxocara*, *Trichinella*. The basis for prevention of emerging zoonotic diseases is the development of precise (unambiguous) methods for identifying pathogens. Before humans get infected, Anisakidae and Raphidascarididae occur in organisms that are intermediate and paratenic hosts, hence understanding the environment of their occurrence and development cycles is a prerequisite for protecting human health. By understanding the mechanisms of invasion in wild animal populations, we will be able to better predict the possible ways of infection of people.

Systematics of nematodes based on morphological and anatomical features underwent multiple changes over the past decades. The reasons were the difficulty in observing complex development cycles, problems in the selection of appropriate criteria and the use of many synonymous names. In addition, many individuals were described in larval stages that are hard to identify because of the low number of differentiating features and small size. With the development of molecular techniques – as the analysis of allozymes or DNA (PCR–RFLP, PCR–SSCP, sequencing) – it was possible to unify the systematic division of nematodes. It has been discovered that many species, previously described only on the basis of morphological features, constitute the complexes of sibling species, which differ genetically and environmentally, and are almost indistinguishable morphologically.

Due to the potential pathogenicity of Ascaridoidea to humans, the aim of my thesis was to investigate the spread of nematodes from this superfamily. I examined, among others, presence of Anisakidae in ringed seal (*Pusa hispida*) and bearded seal (*Erignathus barbatus*) from Hudson Bay (Northern Canada). These phocids, being definitive hosts for *C. osculatum* sensu lato and *P. decipiens* sensu lato, play significant role in the transmission of listed parasites to fish, which in turn are an important food source for the Inuit. In order to determine the risks associated with the consumption of traditional food by the indigenous people of Northern Canada, I tried to state the species composition, level of infection and developmental stages of parasites in the examined phocids.

The main issue of the dissertation was to determine the species composition of nematodes in the examined species of fish and marine mammals and to investigate the dynamics of infection (abundance, prevalence and intensity of infection). In addition, the goal was to determine the correlation between the level of infection and the species, age and gender of hosts. Another analyzed issue was to study the preferences of Ascaridoidea in relation to occupied place in the bodies of fish and differences in infection depending on the region and depth of catch. Where this was possible the stomachs content was determined as the possible way of Ascaridoidea contamination.

The results obtained within this scope of work allow to learn the factors affecting the health condition of selected species of fish and final hosts, which contribute to the spread of parasites in the environment.

The material for my research came from the waters of the North Holarctic, and was acquired through collaboration with numerous scientific institutions. Parasitic nematodes of Greenland halibut *Reinhardtius hippoglossoides* [1] have been collected in cooperation of the University of Gdańsk with Institute of Oceanology Polish Academy of Sciences (IO PAS) during commercial cruise on the Barents Sea. During another voyage I managed, in cooperation with IO PAS and Fisheries Service under the Ministry of Agriculture Division of Fisheries research & science (Klaipeda, Lithuania), to collect parasitological material from fish of great economic importance (including Greenland halibut *Reinhardtius hippoglossoides*, Atlantic cod *Gadus morhua*, redfish *Sebastes mentella*, American plaice *Hippoglossoides platessoides*) [5]. Thanks to the cooperation with the Makivik Corporation (Kuujjuaq, Canada), it was possible to obtain material from ringed seals *Pusa hispida*, bearded seals *Erignathus barbatus* and beluga whales *Delphinapterus leucas* from Northern Canada [2, 4]. In addition, fish from Lake Baikal (the Baikal yellowfin *Cottocomephorus grewingkii* and the longfin Baikal sculpin *Cottocomephorus inermis*) were tested parasitologically, obtained due to the cooperation with the Baikal Museum of Irkutsk Scientific Center of the Siberian Branch of the Russian Academy of Sciences (Listvyanka, Russia) [3].

In the framework of present study for the occurrence of parasitic nematodes examined were: 243 fish (from 5 species) from the Barents Sea, and isolated from them 11,122 individuals of Ascaridoidea [1, 5], 7 bearded and 59 ringed seals from the Hudson Bay infected respectively with 2,650 and 94 nematodes [2], 88 *C. grewingkii* and 35 *C. inermis* from Lake Baikal infected respectively 84 and 51 Anisakidae [3] and from Northern Canada 13 beluga whales, of which 190 nematodes were isolated [4].

In order to prepare the material for identification, the parasites were divided into three parts. Anterior and posterior parts were stored in 70% ethanol with 5% glycerol and examined using a light microscope by evaporation of the ethanol/glycerin mixture. Central parts were prepared for molecular identification by fixing in 70% ethanol.

Nematodes were identified based on morphological and anatomical features. Among others, the presence of boring tooth, mucron, spicules, ventriculus and intestine ceaca, shape of ventriculus and position of the excretory pore were studied. Having in mind that the identification of nematodes based only on differences in morphology does not allow to identify the species within the sibling species complex, the molecular methods were applied: analysis of restriction fragment length polymorphism (PCR–RFLP) of conservative fragment of nuclear ribosomal DNA (rDNA) comprising gene 5.8S and non-coding sequences flanking it (ITS1 and ITS2), and sequencing. PCR–RFLP technique was chosen because of short time of analysis, commonness in the identification of Ascaridoidea, and thus a large number of available molecular keys, and its relatively low cost. Molecular identification of Ascaridoidea superfamily members was based on a comparison of digestions patterns of restriction enzymes with available molecular keys or analysis of nucleotide sequence data from those from the GenBank.

Using a light microscope parasites were qualified to 4 complexes of sibling species: *Anisakis simplex, Contracaecum osculatum, Pseudoterranova decipiens* and *Hysterothylacium aduncum*. Only because of the use of molecular techniques there were possibility to extract the sibling species. Among the studied hosts from North Holarctic 7 species were reported: *Anisakis simplex* sensu stricto, *Contracaecum osculatum* A, *C. osculatum* B, *C. osculatum* C and *C. osculatum baicalensis*, *Pseudoterranova bulbosa* and *Hysterothylacium aduncum*. The biggest prevalence of infection in those sample showed: *C. osculatum* sensu lato and *A. simplex* sensu lato.

In the analyzed material, the highest number, as 84% of the fish surveyed in Barents Sea, constituted the Greenland halibut [1, 5]. This species has been selected for analysis because it is commercially fished and widely consumed in many countries, and as a host of Ascaridoidea may contribute to the dissemination of anisakidosis. In this benthopelagic fish species the dominant parasite was *A. simplex* sensu stricto which accounted for 84% of all noted nematodes from halibuts caught in 2007 [1] and 73% in the sample from 2011 year [5]. The second the most abundant was *Contracaecum osculatum* sensu lato (15% and 21%, respectively). In addition, few individuals of *H. aduncum* and *P. bulbosa* were found. The majority of the nematodes were at L3 larval stage except for *H. aduncum* in sample from 2011 year [5], represented also by L4 and adults.

From halibuts caught in 2007 [1], 98% were infected by Ascaridoidea. There was an increase in the infection along with the halibut's length, as confirmed by literature data about larval accumulation in conjunction with age of fish. Due to the distribution of sea currents in the Barents Sea, the sample was divided into two groups: northern and southern. It was observed that these groups differ significantly in the intensity of infection of halibut by *C. osculatum* sensu lato The intensity of infection in the northern group was higher than in the southern, what imply other food sources in the studied groups. Moreover, different levels of Ascaridoidea infection were reported depending on the depth of catch. Fish from shallow waters (<610m) showed a higher abundance of *A. simplex* sensu lato than those from the deep ones, but in the case of *C. osculatum* sensu lato the trend was reversed. In Greenland halibut, from the *C. osculatum* complex, 3 sibling species were identified: *C. osculatum* A, *C. osculatum* B and *C. osculatum* C, of which the last two species were recorded for the first time in Greenland halibut in the Barents Sea. Mixed infection of *C. osculatum* sibling species were noted.

In subsequent studies of parasitic nematodes from the Barents Sea [5] the research area was expanded: nematodes from 5 fish species (*R. hippoglossoides, S. mentella, G. morhua,* *Macrourus berglax* and *H. platessoides*) were isolated retaining the place of occurrence in the fish body. Nematodes were isolated from the stomach (the wall and the lumen), from intestine, liver, pyloric caeca and body cavity (including gonads and mesentery). Representatives of Ascaridoidea showed different preferences for settling on specific organs. *Anisakis simplex* sensu stricto, the dominant species, was found in the liver, stomach and intestines in similar proportions (29.5%, 29.1% and 28.6%, respectively). *Hysterothylacium aduncum* was the most commonly quoted in intestines and stomach (45.3% and 38.1%, respectively) whereas *C. osculatum* sensu lato clearly preferred the intestines (57.0%). In turn, *P. bulbosa* was the most abundant (45.5%) in the liver.

Various Ascaridoidea infections have been reported in this study, depending on the species of fish and its habitat. *Hippoglossoides platessoides*, typically benthic species, was characterized by high *P. bulbosa* infection, contrary to benthopelagic fish such as Atlantic cod and Greenland halibut. In all fish from the Barents Sea, except *H. platessoides,* the dominant species was *A. simplex* sensu stricto. Again, the differences in Ascaridoidea infections between trials were noted: northern vs southern (division formed due to the distribution of currents in the Barents) and shallow water vs deep for all fish. *Anisakis simplex* sensu stricto and *H. aduncum* showed higher infection in the shallow southern sample while for *P. bulbosa* bigger infestation was recorded in the deep, north waters. Abundance of *C. osculatum* sensu lato did not change with relative to analyzed categories. An analysis of *C. osculatum* distribution in Greenland halibut was performed to compare with previous studies. Three species of *C. osculatum* siblings were observed: *C. osculatum* A, B and C, and 1 specimen – a hybrid of *C. osculatum* B and *C. osculatum* C [5].

Another species of *C. osculatum* complex is *C. osculatum baicalensis*, typical for Lake Baikal. The distribution of this nematode was studied in two species of fish: *C. grewingkii* and *C. inermis*, which are second intermediate hosts for this ascaridoid [3]. There was an increase in the intensity of infection with the size of the fish and differences in the infection between the sex of host.

In addition to analyzing infection levels in intermediate and paratenic hosts, it was intended to examine the infection of Ascaridoidea in the final hosts. For this purpose, the stomachs of bearded and ringed seals from Arviat and Sanikiluaq (Hudson Bay) and from belugas from Northern Canada, areas from which no Ascaridoidea has been previously studied in *D. leucas* (Hudson's Bay and Hudson Strait), were tested. Three species of parasites, *P. bulbosa* and *C. osculatum* A and C, were observed in the phocids, while in belugas: *A. simplex* sensu stricto, *P. bulbosa* and *C. osculatum* A and C. The *Erignathus* *barbatus* specimens were more infected than *P. hispida* (intensity 379 vs 5 specimens, respectively). The relationship between nematode–host species was observed, probably associated with host's dietary preferences and lifestyle. In the bearded seal 100% of the specimens were infected with *P. decipiens* sensu lato whereas *C. osculatum* sensu lato was dominant in the ringed seal. For both species the predominance of adult nematodes over L4 and females over males was observed. The successful of this doctoral dissertation is discovery of *C. osculatum* A and C in the Hudson Bay, as the first record in this region [2].

Among the investigated belugas the highest prevalence and intensity of infection were recorded for *P. decipiens* sensu lato, and the lowest for *A. simplex* sensu lato, although it is considered to be a species typical for cetaceans. This situation may has been caused by a large numbers of phocids in this region, which transmit via the trophic chain specific for them parasites (like *P. decipiens* sensu lato) to belugas. The isolated nematodes represented 3 developmental stages: L3 (dominant), L4 and adult (with a higher proportion of males than females). Interestingly, L4 and adult specimens were recorded only for *P. decipiens* sensu lato, which indicates that the beluga is a final host for this anisakid. This is probably related with diet preferences of the beluga, which feed, among other, on benthic fish and invertebrates, which are listed as intermediate hosts for *Pseudoterranova* genus. This is also confirmed by the present study, where in the stomachs of the beluga whales, hard parts of invertebrates (e.g. jaws of *Nereis* and squid beaks) were found [4].

The results obtained in this work may affect the overall perception of the spread and interaction of nematodes with their environment, which are not only the final hosts, but also the paratenic and intermediate ones, with specific environmental preferences and range of occurrence. Nematodes from Ascaridoidea superfamily are widespread parasites of fish, mammals and fish eating birds, occurring also in polar zones (North Atlantic, Hudson Bay, Barents Sea). For the first time, some species of Ascaridoidea have been reported in new host species in the listed waters [1, 2]. The gained knowledge has not only biological but also medical importance, as it indicates the degree of infection of species which are food source for human and potentially dangerous because of the risk of anisakidosis [1–5].

The results of the analyzes provide an insight into the level of infection of fish species important to the fishing industry. The former problem is the traditional identification of Ascaridoidea and the difficulty in distinguish the larval stages, which are only slightly different morphologically and anatomically, but differ in biology and pathogenicity. The above difficulties are solved in this work by use of molecular techniques (PCR–RFLP, sequencing).

Another interesting issue is the uneven infection of fish with sibling species. This study makes possible to accurately determine the dynamics of infection with sibling species, so that sanitary regulations on monitoring and testing methods for fish raw materials can be updated.

The issues raised during the dissertation are also part of the fifth sector of the national economy, which includes i.a. health care. The widespread popularity for raw fish consumption creates non aware threats to consumers. Since 1960. over 34,000 cases of anisakidosis have been reported, of which around 2,000 are reported in Japan each year and 500 in Europe. It is not difficult to infect, when for example for cod the internationally standard Codex Alimentarius tolerates meat infection for up to 5 nematodes in 1kg of fish. The detailed studies of parasitic nematodes and the degree of infection of the investigated hosts allows to determine the species which consumption is associated with the highest risk of anisakidosis.