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Transmission of lungworms of harbour porpoises and harbour seals: Molecular tools determine potential vertebrate intermediate hosts ☆

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ABSTRACT

Harbour porpoises (*Phocoena phocoena*) and harbour seals (*Phoca vitulina*) from German waters are infected by six species of lungworms (Metastrongyloidea). These nematodes parasitise the respiratory tract, are pathogenic and often cause secondary bacterial infections. In spite of their clinical and epidemiological significance, the life cycle and biology of lungworms in the marine environment is still largely unknown. Regions of ribosomal DNA (ITS-2) of all lungworms parasitising harbour porpoises and harbour seals in German waters were sequenced to characterise and compare the different species. The phylogenetic relationship among the lungworm species was analysed by means of their ITS-2 nucleotide sequences and the species-specific traits of the ITS-2 were used to screen wild fish as possible intermediate hosts for larval lungworms. Molecular markers were developed to identify larval nematodes via in-situ hybridisation of tissues of harbour porpoise and harbour seal prey fish. Potential wild intermediate fish hosts from the North Sea were dissected and found to harbour larval nematodes. Histological examination and in-situ hybridisation of tissue samples from these fish showed lungworm larvae within the intestinal wall. Based on larval ITS-2 nucleotide sequences, larval nematodes were identified as *Pseudalius inflexus* and *Parafilaroides gymnurus*. Turbot (*Psetta maxima*) bred and raised in captivity were experimentally infected with live L1s of *Otostrongylus circumlitus* and ensheathed larvae were recovered from the gastrointestinal tract of turbot and identified using molecular tools. Our results show that fish intermediate hosts play a role in the transmission of metastrongyloid nematodes of harbour porpoises and harbour seals.

1. Introduction

Metastrongyloid nematodes or lungworms are usually associated with the respiratory tracts of mammals (Arnold and Gaskin, 1974; Gosselin and Measures, 1997). In marine mammals they often cause pneumonia, impede diving ability and are an important factor in the health of wild populations (Measures et al., 1995; Gulland and Beckmen, 1997; Siebert et al., 2001). Secondary bacterial infections and inflammatory lesions due to lungworms are a frequent cause of mortality in odontocetes (Gibson et al., 1998; Wünschmann et al., 2001; Lehnert et al., 2005) and pinnipeds (Dailey, 1970; Onderka, 1989; Siebert et al., 2007). In harbour porpoises (*Phocoena phocoena*) from German waters, four species of lungworms occur, all belonging to the Pseudaliidae (Metastrongyloidea), a taxon restricted almost entirely to toothed whales. These species (*Pseudalius inflexus*, *Torynurus convolutus*, *Halocercus invaginatus*, *Stenurus minor*) inhabit different niches in the respiratory tract. Some species, such as *S. minor* in harbour porpoises (*P. phocoena*) and *Stenurus globicephalae* in pilot whales (*Globicephala melas*), also invade the tympanic bullae and cranial sinuses (Arnold and Gaskin, 1974; Faulkner et al., 1998). In harbour seals (*Phoca vitulina*), two lungworm species occur (*Otostrongylus circumlitus*, Crenosomatidae; *Parafilaroides gymnurus*, Filaroididae). Despite the effect of lungworms and associated diseases on the health of their host populations, descriptive studies on parasite load predominate the literature (e.g., Onderka, 1989; Bergeron et al., 1997a; Gulland and Beckmen, 1997; Gosselin et al., 1998; Jepson et al., 2000; Houde et al., 2003b).

Lungworms infecting marine mammals have evolved from ancestors infecting terrestrial hosts, and the survival of the ancient family Pseudaliidae in marine hosts is assumed to be due to the inability of more modern competitors to arise in the marine

☆ *Note:* Nucleotide sequence data reported in this paper are available in GenBank under the Accession Nos.: AY491979, AY464532, FJ767935, FJ787301, FJ787302, FJ787303, FJ787304.

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environment (Anderson, 1982). Although a long period of co-evolution between these marine hosts and their lung parasites can be assumed (Anderson, 1982), currently little is known about the ecology, phylogeny and transmission of lungworms in the marine environment. Most terrestrial metastrongyloids are heteroxenous, using invertebrates as intermediate hosts (Anderson, 2000). Experimental infections using metastrongyloids infecting marine mammals have indicated that they may use a vertebrate as intermediate host (Dailey, 1970; Bergeron et al., 1997b; Houde et al., 2003a) while other studies have found evidence for prenatal infections with lungworms in cetaceans (Dailey et al., 1991). The identification of nematode eggs and larvae to the species level is rarely possible based on morphology unless supported by experimental work on their development or validation using molecular tools.

In addition, several molecular studies have demonstrated the existence of genetically distinct but morphologically similar (cryptic) species (Beveridge et al., 1993; Chilton et al., 1995; Mattiucci et al., 2007). The second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA) provides reliable genetic markers for specific identification of nematodes (Campbell et al., 1995; Stevenson et al., 1995; D'Amelio et al., 2000; Samson-Himmelstjerna et al., 2002; Nadler et al., 2005) and can therefore be used for species identification and for non-invasive diagnosis of parasite infections using faeces or blowhole swabs from live marine mammals, e.g., those undergoing rehabilitation.

The aim of the present study was to characterise the ITS-2 region of the rDNA of seven lungworm species from harbour porpoises, harbour seals and pilot whales to be able to discriminate between species, to analyse the phylogenetic relationship among them and to identify intermediate fish hosts.

2. Materials and methods

2.1. Collection of adult parasites

Lungworms were collected during necropsies performed on stranded harbour porpoises and porpoises killed as bycatch, as well as stranded harbour seals from the Baltic and North Seas off Germany. All were examined as part of monitoring programmes to evaluate their health status (Siebert et al., 2007). Some animals found terminally ill were killed by gunshot by authorised national park rangers or by lethal injection by a veterinarian (Lehnert et al., 2007). Lungworm parasites were identified based on morphological characteristics of adults (Lehnert et al., 2005, 2007). Animals were dissected after storage at –20 °C for up to 6 months however a few were dissected while fresh. Parasites were preserved in 70–90% ethanol or frozen at –20 or –70 °C. DNA was isolated from adult specimens of *P. inflexus*, *T. convolutus*, *H. invaginatus*, *S. minor* infecting harbour porpoises, and from adult specimens of *O. circumlitus* and *P. gymnurus* infecting harbour seals. Lungworms came from harbour porpoises and harbour seals of different age classes, both sexes and from the North and Baltic Sea. Adult specimens of *S. globicephalae* (Pseudaliidae, Metastrongyloidea) came from a pilot whale (*G. melas*) stranded on the North Sea coast of Schleswig–Holstein, Germany. Voucher specimens have been deposited in the Senckenberg Institute, Forschungsinstitut und Naturmuseum Frankfurt, Frankfurt, Germany (Accession Nos. SMF 16897–16903).

2.2. Collection of larval parasites

Fish were obtained fresh as bycatch from a ship trawling for shrimp in the German North Sea. Trawls came from three different areas within the North Sea: Sylt Outer Reef (6° 54′ E; 54° 57′ N), west of the island of Helgoland (7° 44′ E; 54° 12′ N) and close to the mouth of the Eider River (8° 25′ E; 54° 12′ N).

Of the 188 fish examined, 99 (53%) were plaice (*Platessa pleuronectes*), 55 (29%) were dab (*Limanda limanda*) and the rest consisted of rockling (*Gaidropsarus vulgaris*) ($n = 1$), dragonet (*Callionymus lyra*) ($n = 1$), grey gurnard (*Eutrigla gurnardus*) ($n = 1$), solenette (*Buglossidium luteum*) ($n = 10$), scaldfish (*Arnoglossus laterna*) ($n = 3$), sandeel (*Ammodytes tobianus*) ($n = 14$), flounder (*Platichthys flesus*) ($n = 2$) and lemon sole (*Microstomus kitt*) ($n = 2$). These fish were selected because they are important prey items of harbour porpoises and harbour seals in the North and Baltic Seas (Sievers, 1989; Benke et al., 1998; Gilles, A., 2009. Characterisation of harbour porpoise (*Phocoena phocoena*) habitat in German waters. Dissertation (doctoral thesis). Christian-Albrechts-Universität zu Kiel, http://eldiss.uni-kiel.de/macau/receive/dissertation_diss_00003429). Fish ranged in size from 13 to 25 cm in length and were dissected and the gastrointestinal tract opened. After removing its contents, the tissue was compressed between two Petri dishes and examined for parasites using a binocular microscope (Olympus SZH10 Research Stereo). Measurements of larvae were made using a digital camera (Olympus Camedia) attached to the binocular microscope with Olympus DP-Soft Version 3.2 software on a personal computer. Live larvae were isolated from the intestinal wall or attached adipose tissue using forceps and needles prior to DNA isolation.

2.3. DNA isolation, amplification and sequencing

Genomic DNA from individual adult worms or individual larvae was isolated using a QIAamp Tissue Kit or QIAamp Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The rDNA ITS-2 was amplified from five to 15 individual adult worms per nematode species. The nucleotide sequences were aligned and a consensus sequence established. For each individual worm, PCR and nucleotide sequencing reactions were performed at least twice. rDNA ITS-2 was amplified by PCR using primers designed from the adjacent conserved 5.8S and 28S regions of *Caenorhabditis elegans* (GenBank Accession No.: X03680). Oligonucleotide primers were: 5′-GCA GAC GCT TAG AGT GGT GAA A-3′ and 5′-ACT CGC CGT TAC TAA GGG AAT C-3′. The PCR started with an initial step at 94 °C for 3 min, followed by 39 cycles of: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min. It ended with a 5 min step at 72 °C. Primer concentrations were 20 pmol/μl and Taq polymerase (Applied Biosystems, Darmstadt, Germany) was used.

Nucleotide sequencing reactions were performed either using DYEnamic ET terminator sequencing chemistry (Applied Biosystems, Darmstadt, Germany), PCR products were directly sequenced using a MegaBACE 1000 capillary sequencer or were outsourced (Seqlab Göttingen). Some rDNA ITS-2 regions showed repeats in their structure and these PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) and a pCR4-TOPO vector to facilitate sequencing. Plasmid DNA was isolated using a NucleoSpin Kit (MachereyNagel, Düren, Germany) and then sequenced. Nucleotide sequences were edited and aligned using DNAstar (version 5.07/5.52) software. As a control, 18S regions were amplified and sequenced from larvae encountered in the fish and negative controls without template were included in PCR reactions. Primer sequences delineated from *C. elegans* (Accession No.: X03680) were 5′-CCC GAT TGA TTC TGT CGG C-3′ and 5′-GGC TGC TGG CAC CAG ACT TGC-3′.