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Lungworm seroprevalence in free-ranging harbour seals and molecular characterisation of marine mammal MSP

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ABSTRACT

Harbour seals (*Phoca vitulina*) are frequently infected with the lungworms *Otostrongylus circumlitus* and *Parafilaroides gymnurus*. The infection is often accompanied by secondary bacterial infections and can cause severe bronchopneumonia and even death in affected animals. Hitherto, the detection of lungworm infections was based on post mortem investigations from animals collected within stranding networks and a valid detection method for live free-ranging harbour seals was not available. Recently, an ELISA was developed for detecting lungworm antibodies in harbour seal serum, using major sperm protein (MSP) of the bovine lungworm, *Dictyocaulus viviparus* as recombinant diagnostic antigen. To determine lungworm seroprevalence in free-ranging harbour seals, serum was taken from four different seal age groups (n = 313) resulting in an overall prevalence of 17.9% (18.9% of males, 16.7% of females). 0.7% of harbour seals up to six weeks of age were seropositive, as were 89% of seals between six weeks and six months, 53.6% between six and 18 months and 24.2% of seals over 18 months of age. In the 18 months and over age group, seropositive animals showed statistically significant reductions in body weight ($P = 0.003$) and length ($P < 0.001$). Sera from lungworm infected harbour seals in rehabilitation (n = 6) revealed that duration of antibody persistence may be similar to that of lungworm infected cattle, but further studies are needed to confirm this. Phylogenetic analyses of MSP sequences of different marine and terrestrial mammal parasitic nematodes revealed that lungworm MSP of the genus Dictyocaulus (superfamily Trichostrongyloidea) is more closely related to metastrongylid marine mammal lungworms than to trichostrongylid nematodes of terrestrial hosts.

1. Introduction

After the first seal epidemic occurred in 1988/89, caused by a phocine distemper virus, a network along the coasts of the German Federal State Schleswig–Holstein was established to monitor stranded marine mammals. During necropsy of stranded harbour porpoises and harbour seals findings frequently showed nematodes including lungworms *Otostrongylus circumlitus* (Crenosomatidae) and *Parafilaroides gymnurus* (Filaroididae) of harbour seals (Lehnert et al., 2005, 2007; Siebert et al., 2007). *O. circumlitus* was found in the bronchi, in the right heart chamber, the *Vena pulmonalis* and in the blood vessels of the liver (De Bruyn, 1933; Onderka, 1989; Claussen et al., 1991; Measures, 2001; Lehnert et al., 2007), while *P. gymnurus* mainly parasitised the alveoles and bronchioles (Stroud and Dailey, 1978; Claussen et al., 1991). Varying lungworm prevalence, up to 76%, was reported in harbour seals found in the German Wadden Sea (Claussen et al., 1991; Lehnert et al., 2007; Siebert et al., 2007). Infections were age-related, with most infections occurring in young animals between two and 18 months of age. Harbour seals may start acquiring lungworm infections after nursing for four weeks and after a post-weaning fast of 15–17 days (Muelbert and Bowen, 1993; Ross et al., 1994) when they start to consume prey species (Measures, 2001). Benthic fish were identified as potential intermediate hosts of lungworms (Dailey, 1970; Bergeron et al., 1997a; Lehnert et al., 2010); however, the complete life cycle is yet unknown.

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Lungworms in harbour seals can cause severe pathological changes, like obstruction of bronchial tubes, and are often accompanied by bacterial infections leading to severe bronchopneumonia and death (Measures, 2001; Lehnert et al., 2007; Siebert et al., 2007; Rijks et al., 2008). Clinical symptoms include bronchospasm, anorexia, dehydration, and individual *O. circumlitus* specimens can be observed in sputum (Bergeron et al., 1997a; Measures, 2001). Lungworm infections are mainly reported from stranded harbour seals during post mortem examinations (Claussen et al., 1991; Siebert et al., 2007; Rijks et al., 2008), but those data can be biased by different influences such as age, diseases and anthropogenic activities (Claussen et al., 1991; Measures, 2001; Siebert et al., 2007). Diagnosis in living seals is difficult, as detecting lungworm larvae in faeces has limited sensitivity (Schnieder, 1992). Collecting harbour seal faeces is logistically challenging and assigning samples to individual free ranging seals is not feasible. Due to the difficulties in diagnosing lungworm infections in living seals, prevalence data in the free-ranging harbour seal populations is missing. Therefore, an existing ELISA for immunodiagnosis of the bovine lungworm *Dictyocaulus viviparus* (Schnieder, 1992; von Holtum et al., 2008) was adapted to harbour and grey seals with a resulting sensitivity of 98% and a specificity of 100% (Ulrich et al., 2015). The ELISA represents a reliable method for diagnosing lungworm antibodies in serum samples of free-ranging harbour seals. Recombinant major sperm protein (MSP), a protein family occurring in nematode sperm only (Klass and Hirsh, 1981; Ward et al., 1988) serves as diagnostic antigen.

Information about the molecular structure of MSP from nematodes infecting harbour seals and harbour porpoises is missing. Previous phylogenetic analyses within the Metastrongyloidea have been performed on the base of large-subunit and small-subunit ribosomal (r)RNA (Carreno and Nadler, 2003), the ITS-2 region of rDNA (Lehnert et al., 2010) and the 18S and 28S rRNA (Chilton et al., 2006). Those analyses confirmed the close relationship of marine mammal lungworms within their superfamily Metastrongyloidea, an evolutionary old group that was derived from the terrestrial ancestors of seals and porpoises (Anderson, 1984; Carreno and Nadler, 2003).

The aim of this study was to assess lungworm seroprevalence in free-ranging harbour seals in different age groups. Furthermore, consecutive serum samples of harbour seals in rehabilitation were analysed to obtain first information on the persistence of serum anti-lungworm-MSP antibodies. Additionally, MSP genes from different nematodes infecting harbour porpoises and harbour seals were identified and sequenced to explore phylogenetic relationships between marine and terrestrial parasitic nematodes.

2. Material and methods

2.1. ELISA

2.1.1. Age determination of harbour seals

The approximate age of sampled harbour seals was determined and sorted in age groups, considering sampling date, body-length and body-weight. In young seals, navel and canine development was additionally considered. Age group (AG) 1 included harbour seals from birth to six weeks of age, AG 2 harbour seals from six weeks to six months, AG 3 from six to 18 months and AG 4 above 18 months of age.

2.1.2. Sera of free-ranging harbour seals

All experimental procedures involving harbour seals were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas of the federal state Schleswig Holstein, Germany [permit number: V312-72241.121-19 (70-6/07)], the Danish Nature Agency (SNS-3446-00054 and SN 2001-34461/SN-0005) and the Animal Welfare Division (Ministry of Justice, Denmark, 2005/561-976).

Serum was taken from a total of 313 free-ranging harbour seals. 141 serum samples were taken in June, one sample in May and two samples in July. As 95% of harbour seals are born in June, and nursing takes four weeks, pups sampled in May and June were considered as not weaned (Ross et al., 1994; Abt, 2002). The two animals sampled in July had an unknown weaning status and harbour seals captured in September 2014 were designated as weaned because sampling was conducted when the harbour seals had already finished nursing.

Because of their approximate age, AG 3 and AG 4 animals were considered as weaned regardless of the sampling date. Detailed information on sampled harbour seals is given in Table 1.

Blood was taken with a 1.20 × 100 mm needle (SUPRA, Ehrhardt Medizinprodukte, Geislingen, Germany) from the extradural intervertebral sinus 5 cm cranial to the pelvis (Dierauf and Gulland, 2001), or from the tarsal sinus of the hind flippers (Sanchez Contreras, 2014) and filled in tubes containing serum separation gel. Serum was obtained by centrifugation at 3000x *g* for 15 min and stored at −20 °C until use.

2.1.3. Sera of harbour seals in rehabilitation

Consecutive serum samples were taken for routine diagnostic examinations from six harbour seals at the Seal Rehabilitation and Research Center (SRRC), Pieterburen, The Netherlands (Table 1). The animals were found between January and June 2015 and were suspected or diagnosed to have lungworm infections. *O. circumlitus* specimens were found in sputum in three of six individuals, and all six harbour seals showed symptoms like dyspnoe, coughing and a poor body condition. Samples were taken on three to five different occasions during a period of about 12–19 weeks (Fig. 1). Blood was sampled and processed as described above.

2.1.4. Statistical analyses

Statistical significance of differences between body weight and body length and age distribution within the lungworm infected and negative animals was tested using SigmaStat (version 3.11, Systat Software GmbH, Erkrath, Germany).

2.1.5. ELISA

All sera were tested with the harbour/grey seal adapted recombinant MSP-ELISA as previously described (Ulrich et al., 2015). All samples were analysed in duplicates and the optical density (OD) arithmetic mean of the duplicates was corrected for the blank value. Serum samples were assigned as positive or negative considering the evaluated cut-off value of 0.422 OD (Ulrich et al., 2015).

2.2. Molecular characterisation of MSP in parasitic nematodes of harbour seals and harbour porpoises

2.2.1. Parasite material

Adult nematodes were collected during necropsies of harbour seals and harbour porpoises and their species identified by stereomicroscopic examination (45x magnification; Olympus® SZ 61, Hamburg, Germany). Nematodes of harbour seals included lungworms (*O. circumlitus*, Crenosomatidae: Metastrongyloidea and *P. gymnurus*, Filaroididae: Metastrongyloidea), heartworms *(Acanthocheilonema spirocauda,* Onchocercidae: Filarioidea), intestinal nematodes (*Contracaecum osculatum* and *Pseudoterranova decipiens*, Anisakidae: Ascaridoidea) and lungworms of harbour porpoises (*Pseudalius inflexus*, *Torynurus convolutus, Halocercus invaginatus* and *Stenurus minor*, Pseudaliidae: Metastrongyloidea).