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SHORT COMMUNICATION

Third-stage nematode larvae of *Contracaecum osculatum* from Baltic cod (*Gadus morhua*) elicit eosinophilic granulomatous reactions when penetrating the stomach mucosa of pigs

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Abstract Third-stage larvae of the anisakid nematode *Contracaecum osculatum* were recovered from livers of Atlantic cod (*Gadus morhua*) caught in the Baltic Sea (June 2014) and used for experimental infection of two pigs (one male and one female). Each pig received 215 larvae by oral infection (feeding with minced cod liver containing live nematode larvae). Pigs were euthanized after 5 days, necropsied, and subjected to parasitological investigation. A total of 12 larvae were found penetrating the mucosa of the ventricle (7 in the female pig and 5 in the male pig) eliciting a granulomatous reaction at the penetration site. Four non-attached larvae were found in the female pig stomach and one in the male pig. Petechial bleeding was observed at several locations in the ventricular mucosa where larvae were located. Histological examination of the stomach mucosa revealed a massive cellular infiltration (giant cells, lymphocytes, macrophages, granulocytes, and fibroblast like cells) around the penetrating larva. Mononuclear and polymorphonuclear cells containing eosinophilic granulae were particularly prominent in the granulomas. Reactions correspond to reactions in pigs following experimental infection with the human pathogenic anisakid larvae *Anisakis* sp. and *Pseudoterranova* sp. which suggests that *C. osculatum* might have a zoonotic potential as well.

Keywords Zoonosis · *Contracaecum osculatum* · Experimental infection · Pigs · Fish

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Introduction

Baltic cod (*Gadus morhua*) has recently been reported to be heavily parasitized by third-stage larvae of the anisakid nematode *Contracaecum osculatum* which primarily infects the liver of cod (Haarder et al. 2014; Mehrdana et al. 2014; Nadolna and Podolska 2014). The occurrence has increased from a low frequency in the period from 1982 to 2003 (Valtonen et al. 1988; Perdiguero-Alonso et al. 2008) and has been explained by extensive expansion of the grey seal population in the Baltic Sea during the latest decade (Buchmann and Kania 2012; Haarder et al. 2014; Mehrdana et al. 2014) because these phocids function as final hosts for this parasite. The adult parasite stage occupies the seal stomach from where infective eggs are passed in host feces to the aquatic environment. This complies with the high infection rate found in cod during the 1940s and 1950s when the grey seal population peaked temporarily (Petrushevski and Shulman 1955). Various crustaceans act as intermediate/transport hosts (Køie and Fagerholm 1995), and the cod acquire infection by ingesting these crustaceans. *C. osculatum* larvae reside mainly in the liver of cod although they may occasionally be found in fillet samples (Mehrdana et al. 2014). *C. osculatum* infections have been reported in patients following ingestion of Baltic fish (Schaum and Müller 1967) and fish from Japanese waters (Nagasawa 2012) which strongly suggests that *C. osculatum* larvae may have a zoonotic potential if ingested with raw or under-cooked fish products. Related anisakid nematodes such as *Anisakis* spp. (Smith and Wootten 1978; Audicana and Kennedy 2008; Mattiucci et al. 2013; Baird et al. 2014) and *Pseudoterranova* spp. (Margolis 1977; Pinel et al. 1996; Torres et al. 2007) are well recognized human pathogens. Experimental infection of laboratory animals (such as rats, dogs, and rabbits) have been

performed to characterize pathological reactions in association with *Anisakis* and *Pseudoterranova* infections (Margolis 1977; Smith and Wootten 1978). Histopathological reactions studied by Bier et al. (1976) at the site for penetration of these larvae in the pig stomach mucosa included prominent representation of eosinophilic granulocytes. Experimental infections using third-stage *C. osculatum* larvae have previously been conducted with rats (Fagerholm 1988) who demonstrated an ability of the worm larvae to penetrate the stomach wall of rats. The present study on experimental infection of pigs with *C. osculatum* third-stage larvae recovered from Baltic cod livers has been performed in order to elucidate if this nematode species can infect a pig host and describe any pathological and histopathological reactions elicited by the infection. By comparing these reactions with previously described reactions in pigs with well-recognized zoonotic anisakid nematode larvae, it may enable us to evaluate a possible zoonotic potential of *C. osculatum* third-stage larvae from Baltic cod.

Materials and methods

Third-stage nematode larvae of *C. osculatum* were recovered from livers of Baltic cod (body length 40–60 cm) caught (June 18, 2014) in the Baltic sea immediately east of the island of Bornholm. Livers were removed from cod by gutting immediately after catch and transported under cooled conditions to our laboratory in Copenhagen within 24 h. Individual livers contained between 2 and 30 worms, and a total of 430 live larvae were detected by glass-plate compression technique (Buchmann 2007) and gently picked by a pair of forceps after mechanical separation of liver tissue by hand and divided into two equal portions of 215 worms for experimental infection of two pigs. Worms (both directly taken from cod livers and those recovered after infection of pigs) were examined under a Leica MZ95/MZ12 (Leica Microsystems, Nussloch, Germany) for confirmation of genus based on morphology (of anterior and posterior worm parts) according to Möller and Anders (1986) and Valter et al. (1982). Molecular species determination was performed on a subsample of 21 larvae as described by Mehrdana et al. (2014). Briefly, parasites (mid-body parts) in ethanol (96 %) (Kemetyl, Køge, Denmark) were subjected to lysis for DNA recovery whereafter PCR was performed for amplification and sequencing of the ITS region. Primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) were used as forward and reverse primer, respectively. Identification was based on sequencing 964 bp of the internal transcribed spacer region (3′ end of 18S rRNA, ITS-1, 5.8S rRNA, ITS-2, and 5′ end of 28S rRNA gene). PCR products were purified using the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced at MacroGen (Korea). Gene sequences were aligned and compared with published GenBank sequences using the CLC Main Workbench 7.5 (Qiagen, Denmark).

Two uninfected pigs (one male and one female) were used for the experiment: They were of the D-LY breed (Duroc × Landrace-Yorkshire) weighing 25 kg corresponding to an age of 10–12 weeks. The pigs arrived 1 week prior to the start of the experiment and were delivered from the breeder Niels Ågård, Nyrupsvej 76, 4180 Sorø, CHR: 10169. Before and after infection, the two pigs were kept in a pen together and had access to water and were fed restrictively with a feed mix consisting of 75 % barley and 25 % nutritional supplements (NAG, Helsinge DK) (Hansen, et al. 2014).

Individual pigs were each fed a portion of 215 larvae on June 20, 2014. Worms were mixed with minced Baltic cod liver tissue recovered during the worm isolation, and the mixture was offered each pig in a feeding bowl made of plastic. After feeding, both bowls were rinsed with water and the fluid was examined for larvae. Five larvae were found left over in the male pig's bowl, and no larvae were found in the female pig's bowl. The two pigs were observed every hour during the first 8 h for clinical signs such as vomiting, coughing, diarrhea, or behavioral changes. Pigs were euthanized 5 days after the start of the infection experiment. They received an injection with Zoletil® (tiletamin + zolazepam) and Cepetor Vet. (medetomidin) whereafter they were euthanized with 10 ml pentobarbital (1 %) intracardially.

Pigs were then necropsied, the gastrointestinal canal was removed and the abdominal cavity was flushed with tap water. The rinsing fluid was inspected for larvae, and the liver, lungs, and spleen were inspected for signs of migrating larvae. The esophagus was cut open and inspected. The ventricle was opened and the contents were flushed out and transferred to a 500-μm sieve. The mucosa of the ventricle was inspected for penetrating larvae, their locations noted, and tissue samples with worms were fixed in neutral formalin (formaldehyde solution. 4 % "Lillie" (10 % NBF)) (Hounisen Laboratorieudstyr A/S, Denmark). Nematodes found free in the lumen were transferred to 70 % ethanol for later identification. The duodenum, jejunum, colon, and rectum were cut open, and contents and mucosal surface were inspected for larvae.

Histology was performed on tissue samples (pig stomach mucosa with penetrating worm larvae) which immediately after excision were fixed in neutral formalin, after 24 h transferred to 70 % ethanol and stored for 14 days before processing. Samples were prepared for histology by standard techniques (Buchmann and Kania 2012) and embedded tissues were cut into 4 μm sections using a Leica RM 2135 BioCut Rotary Microtome (Leica Microsystems, Nussloch, Germany). Histological slides were then de-paraffinized and stained either by (1) Mayer's hematoxylin (Dako, Denmark) combined with eosin-solution (Sigma Aldrich, Denmark), (2) Giemsa-azur-eosin-methylene blue solution (Merck

Millipore, Denmark), or (3) toluidine blue solution (Sigma Aldrich, Denmark) for differentiation of mast cells versus eosinophilic cells. Slides stained with Mayer's hematoxylin alone were mounted with the hydrophilic mounting medium Aquatex®. All other slides were dehydrated by immersion in a series of increasing ethanol concentrations (70, 96, and 99 %) and xylene before they were mounted in lipophilic mounting medium DePeX (Bie & Berntsen, Denmark). Histological slides were examined using a Leica DM5000 B light microscope (Leica Microsystems, Nussloch, Germany).

Results

All larvae from the two samples taken from cod liver tissue and from the pig stomachs were identified by morphometric methods as belonging to the genus *Contracaecum* by presence of intestinal and ventricular appendages, pointed caudal part, and location of the excretory pore located anteriorly to the nerve ring. The 21 ITS sequences obtained from *C. osculatum* worms recovered from Baltic cod livers were submitted to GenBank and obtained accession number KM516322 to KM516342. Molecular comparison of the 21 ITS sequences showed 100 % similarity to *C. osculatum sensu stricto* as described by Zhu et al. (2000) and Mehrdana et al. (2014). Both pigs exposed to *C. osculatum* infection by oral administration became infected by the procedure. A total of 12 larvae were found penetrating the mucosa of the ventricle (7 in the female pig and 5 in the male pig). Eleven of the embedded larvae were located in the corpus ventriculi, and one was located in the pars pylorica. It was noted that only the anterior parts (20–30 %) of the larvae were embedded in the mucosa (Fig. 1). Four non-attached larvae were found in the female pig stomach and one in the male pig. Thus, out of 215 larvae ingested by the female pig, a total of 11 worms (5.1 %) survived and thrived in the host. Out of 210 larvae ingested by the male pig (215 offered minus 5 left in the feeding bowl), a total of 6 worms (2.8 %) survived. No larvae were found in the esophagus, duodenum, jejunum, ileum, colon, caecum, rectum, or in the abdominal cavity, and no signs of migrating larvae were observed in liver, lungs, or spleen. The penetrating larvae were found associated with a marked focal swelling around the penetrating larva. Petechial bleeding was observed at several foci in the ventricular mucosa where larvae were located. No clinical signs of infection or behavioral changes of pigs were observed during the experiment, and the hosts showed no signs of reduced feed intake. Histological examination revealed a massive cellular infiltration centered around the penetrating larva (Fig. 2). Giant cells, lymphocytes, macrophages, granulocytes, and fibroblasts were observed around the worm. Mononuclear and polymorphonuclear cells containing eosinophilic granulae were particularly prominent in the granulomas. Differential staining was performed by staining with toluidine blue. If mast cells were present, they would appear red with toluidine blue staining. However, no such metachromasia was detected at all which confirms the presence and identity of eosinophilic granulocytes.

Fig. 1 *Contracaecum osculatum* third-stage larva penetrating stomach mucosa of pig. Anterior 25 % of the larva has penetrated the mucosa which shows swelling at the site of penetration. Total length of larva is 25 mm

Discussion

The present study confirmed that *C. osculatum* third-stage larvae possess the ability to penetrate the stomach mucosa of pigs following oral administration. Massive cellular infiltration was observed around the larva in the stained tissue and large part of the cells contained eosinophilic granules. Similar results have been reported by Bier et al. (1976) studying histological sections of stomach mucosa from pigs experimentally infected with *Anisakis* sp. and *Pseudoterranova* sp. larvae. Eosinophilic and neutrophilic granulocytes have also been

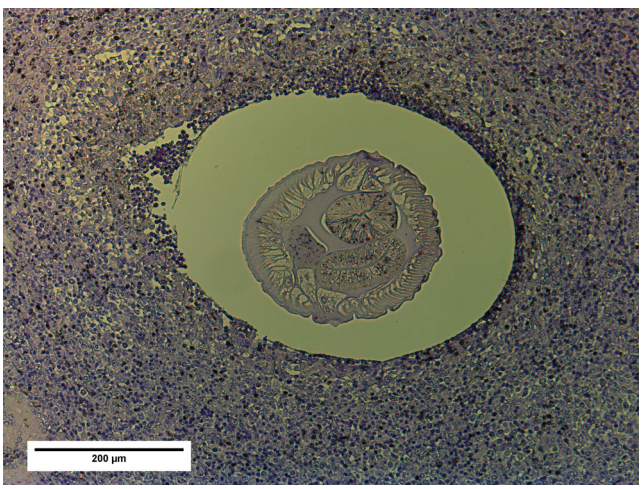

Fig. 2 Transverse section of anterior end of *Contracaecum osculatum* third-stage larva penetrating stomach mucosa of pig. Staining by hematoxylin. Scale bar 200 µm

observed around *Pseudoterranova and Anisakis* sp. larvae in rabbits, rats, dogs, and other experimental animals (see reviews by Margolis 1977; Smith and Wootten 1978). Several *C. osculatum* larvae were found free in the lumen of the ventricle, and it is not known if these larvae had tried to penetrate or simply had detached from penetration site. It cannot be excluded that larvae penetrate the mucosa in one location and then detach and penetrate in a new location. This would explain injuries observed in the present study where numerous petechial hemorrhages were recorded in the stomach mucosa and correspond to worm behavior in experiments where pigs have been infected with *Anisakis* sp. and *Pseudoterranova* sp. (Bier et al. 1976). Third-stage larvae of *C. osculatum* may develop to the L4 stage in the ventricle of rats (Fagerholm 1988), but we only detected third-stage larvae in pigs 5 days post-infection. In conclusion, it can be stated that *C. osculatum* third-stage larvae from Baltic cod livers possess the ability to penetrate the stomach mucosa of pigs where they are associated with hemorrhages and eosinophilic granulomatous reactions in the penetrated tissue. All reactions correspond to previous reports from experimental pig infections with the notorious zoonotic anisakid larvae of the genera *Pseudoterranova* and *Anisakis*, and this may suggest that *C. osculatum* has a zoonotic potential as well.

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