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ORIGINAL PAPER

Alaria alata mesocercariae in raccoons (*Procyon lotor*) in Germany

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Abstract *Alaria alata* is a trematode of carnivores from Europe. The mesocercarial stage was recently identified in wild boar meat from Europe. Previous histopathologic studies showed the presence of unidentified parasitic cysts within the tongues of raccoons from northern Germany. For identification of the parasite species, tissue samples of 105 raccoons originating from a National Park in northern Germany and from Berlin metropolitan area were collected. Histological examination of cryotome sections of frozen as well as paraffin-embedded tongues were used to identify parasite cysts. These were located in the connective and adipose tissue and in close proximity to small arterioles, suggesting a hematogenous spread of the parasite. Often, cysts were surrounded with mild infiltration by inflammatory cells. Additionally, mesocercariae were isolated from defrosted tongue samples of 11 raccoons. Molecular-biology assays confirmed the parasite species as *A. alata*. Except for one positive raccoon from Berlin City, all other positive raccoons originated from the sylvan Müritz National park, indicating an abundance of intermediate hosts in this area. Our results show that raccoons can act as paratenic hosts for *A. alata* and extend the broad host range of this parasite to a species introduced into Germany.

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Abbreviations

Bp	Base pairs
DNA	Deoxyribonucleic acid
HE	Hematoxylin–eosin
ID	Identification
LR	Lewitz region
MNP	Müritz National Park
NTC	Nontemplate control
Syn	Synonym

Introduction

Alaria alata is a parasitic trematode from the family Diplostomatidae and the genus *Alaria*. Its adult stage can be found in the small intestine of carnivores like red foxes (*Vulpes vulpes*), raccoon dogs (*Nyctereutes procyonoides*), European wolves (*Canis lupus lupus*), and domestic dogs from Europe (Borgsteede 1984; Machnicka et al. 2003; Möhl et al. 2009; Murphy et al. 2012). Recently, it was also reported in a domestic cat and a pampas fox (*Lycalopex gymnocercus*) from South America (Castro et al. 2008; Ruas et al. 2008). Adult parasites measure 2.5–6×0.5–2 mm, with the anterior part of the body being wider and larger than the posterior part; measurements for *A. alata* eggs range between 98–125×62–81 μm (Travassos et al. 1969) and 0.56–0.93×0.55–0.77 mm for the mesocercariae (Andreas 2006). The known species of *Alaria* are *A. mustelae*, *A. intermedia*, *A. marcianae*, *A. arisaemoides*, *A. canis* (syn. *A. americana*), and *A. taxideae*, which are found in North and South America (Möhl et al. 2009); while *A. alata* is native to Europe. The definitive hosts were thought to be members of the family Canidae, but current studies also show other carnivores like Felidae; Mustelidae can serve as final hosts as well (Möhl et al. 2009). In the life cycle of this parasite, unembryonated eggs pass through the

final host's feces into water, where miracidia hatch after 2 weeks and penetrate the first intermediate host: fresh water snails (*Planorbis*-, *Heliosoma*-, *Lymnea*-, and *Anisus* species) (Möhl et al. 2009). After a year and two generations of sporocysts, tailed cercariae (furocercariae) are released into the water. Amphibian species are the second intermediate host, where cercariae develop into mesocercariae. The final host is reached by ingestion of an infected intermediate host; once the definitive host is entered, mesocercariae migrate to the lungs and evolve into metacercariae, which migrate to the intestines to reach the adult phase. At the level of the mesocercarial stage and the second intermediate host, the life cycle can be extended to a paratenic host. Here, the mesocercariae remain in an encysted resting phase.

The mesocercariae of *A. alata* are known to infect a number of paratenic hosts (Odening 1963; Möhl et al. 2009). It was also reported in a grass snake (*Natrix natrix*) from Romania, although no description of the developmental stage or the anatomic location was provided (Mihalca et al. 2007). Recently, *A. alata* was increasingly found during routine inspections for *Trichinella* spp. in wild boar meat (*Sus scrofa*) in Germany, France, and Croatia (Jaskšić et al. 2002; Portier et al. 2011; Riehn et al. 2011, 2012), while in the past, occasional findings were reported in pork and beef (Odening 1963). Reports of human alariosis were published for the American species of the genus *Alaria* (Möhl et al. 2009; Fried and Abruzzi 2010) with one fatal case where probably the consumption of undercooked wildlife such as frog legs, raccoon, or wild goose was the source of infection (Fernandez et al. 1976; Beaver et al. 1977; Kramer et al. 1996; Fried and Abruzzi 2010). As for the European *A. alata* Odening (1963) showed severe infestation in an experimentally infected rhesus macaque (*Macaca mulatta*), proving that a host closely related to humans can become infected with this trematode. Besides this information, no human cases of alariosis have been reported in Europe. However, it is evident that a zoonotic risk can be presumed and a potential danger of this parasite has to be taken into consideration (Portier et al. 2011). In Germany, the Federal Institute for Risk Assessment (BfR) considers wild boar meat infected with *A. alata* mesocercariae not suitable for human consumption (BfR 2007) and follows an evaluation of the Swiss Agency for the Environment, Forests, and Landscape (SAEFL) that classified *A. alata* as a zoonotic parasite (Anonymous 2003).

The North American raccoon (*Procyon lotor*) was introduced in Germany almost 80 years ago. It is now widely distributed in several European countries with the highest population density still located in Germany. Here, the raccoon population is rapidly increasing and the animals are declared as game in 14 of the 16 German federal states (Michler and Michler 2012). Raccoons are easily adaptable to wild, rural, and urban habitats and because of their invasive species status, they are gaining importance as potential vectors for infectious and zoonotic diseases in Europe (Beltrán-Beck et al. 2011). The raccoons' habitats overlap with the range of other animal species serving as hosts for *A. alata* such as amphibians, wild boars, red foxes, badgers (*Meles meles*), or raccoon dog (*N. procyonoides*). Although the presence of *A. alata* in raccoons has not been published so far, they have been reported as paratenic and final hosts of *A. mustelae* and *A. marcianae* in North America (Johnson 1979; Shoop and Corkum 1981). To investigate the possible occurrence of *A. alata* in raccoons from Germany, we analyzed tongue tissues of hunted and road-killed animals from northern Germany as well as from Berlin greater metropolitan area.

Material and methods

Study area

Müritz National Park (MNP) and Lewitz region (LR) are located in the federal state of Mecklenburg-Western Pomerania in northeastern Germany. The MNP (322 km^2) is divided into two sections, Müritz (53°26′11″N, 12°50′12″E) and Serrahn (53°21′N, 13°12′O), and it consists mostly of old broadleaf tree forest and widely ramified peat bogs and swamps. As for LR (120 km^2, 53°26′52″N, 11°36′24″O) forests, meadows, and waterways dominate the area. Both locations are rich in wildlife fauna and are visited by tourists all year and by hunters during hunting season.

Berlin is the capital and largest city of Germany (52°30′2″N, 13°23′53″E) with 3.3 million inhabitants, and besides its urban character, numerous natural parks are distributed across the city, while the outskirts are covered with woodlands and lakes. Animal species like wild boars, wild rabbits (*Oryctolagus cuniculus*), red foxes, and raccoons are part of the city's urban wildlife.

Sample collection and histological examination

One hundred and five hunted and road-killed raccoons were collected and analyzed; 88 animals originating from MNP were collected between 2006 and 2011. Six hunted raccoons from LR and 11 road-killed raccoons from Berlin were investigated additionally. The overall sex ratio was 60 males (57.1 %) to 45 females (42.8 %). Adults represented 50.4 % (n=53) of all sampled carcasses. A necropsy was performed on each carcass and samples of tongue, liver, lung, heart, spleen, kidney, brain, intestines, lymph nodes, and reproductive organs were extracted for histological examinations but were not part of this study. For this study, a slice of tissue was taken from the base of each animal's tongue for histopathological examination as previous investigations had identified this location with the highest chance to detect the parasitic cysts in question (unpublished data, G. Wibbelt). Samples

were fixed in 4 % formalin, processed routinely, and embedded in paraffin. Tissue slides were sectioned at 4 μm, stained with hematoxylin–eosin (HE), and examined by light microscopy. Tongues with suspicious parasite cysts were chosen for further analysis. Characteristic histological features of the cysts were recorded, and measurements of the cysts' diameter were taken by microscopic photographs using the software Cell^{A} Olympus SIS (Münster, Germany).

Cryotome sections

Five frozen tongues with high number of suspicious parasite cysts identified in HE sections were cut in serial sections (25 μm) with a cryotome and mounted on glass slides for light microscopy examination. Slides with distinct parasite structures (mesocercariae) within these cysts were selected for DNA extraction to ensure positive raccoon samples.

Parasite identification

For parasite identification, two different sets of samples were used (1) Defrosted tongue tissues were mashed and diluted in warm water to isolate mesocercariae using the *A. alata* mesocercariae migration technique as described by Riehn et al. (2010). (2) Cryotome sections were cut from frozen tongue tissues with histologic evidence of suspicious parasites cysts. Sections with encysted parasite-like structures were selected for molecular identification. Additionally, one negative histological sample was included.

DNA extraction and PCR analysis

Genomic DNA extraction from isolated mesocercariae as well as parasite structures isolated from cryotome sections was performed using QIAamp® Mini Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. After genomic DNA was purified, the molecular identification was carried out by PCR analysis. For the amplification of a selected part of the purified DNA oligonucleotide primers DME-F 5′-CTTAGCTGCGGGTTCCTGCT -3′ and DME-R 5′- CGGC ACATAAGCAAATACCTCG -3′ were used according to Riehn et al. (2011). As positive control, an *A. alata* specimen which was previously identified by the German Federal Institute of Risk Assessment was used. For negative controls, we used a nontemplate control (NTC).

PCR amplicons were separated electrophoretically in 1.5–2 % agarose gel (PEQLAB, Erlangen, Germany) and visualized on a Multi Image® Light Cabinet UV transilluminator (Alpha Innotech, distribution by Biozym Scientific, Hessisch Oldendorf, Germany). Detection of a single band at approximately 300 bp allowed identification of *A. alata* mesocercariae as the specific oligonucleotide primers DME-F and DME-R deliver a band of 303 bp.

Results

Histological examination of paraffin and cryotome sections

From the 105 carcasses collected, 35 animals (33.3 %) had parasite cysts in the examined basis of their tongues. Mild local inflammatory response was found in 28 of these raccoons (77.7 %). Leukocytic infiltrations comprised, in deceasing order, lymphocytes, plasma cells, eosinophilic granulocytes, and neutrophilic granulocytes. Occasionally, some macrophages and multinucleated giant cells were also present. Cysts were detected in the connective and adipose tissue between bundles of myofibers, most often in close proximity to a small arteriole. The cysts had an elongated oval shape with a diameter ranging from 84×115 to 1,170×1,176 μm. Characteristically in most of the samples, a fine basophilic veil was present within the lumen of the cyst (Fig. 1). Besides local inflammation, no other pathological findings including myositis, necrosis, or muscular fibroplasia was found in the tongue samples.

Positive tongue sections contained one parasite cyst in 15 cases (42.8 %), two to three cysts in 9 cases (25.7 %), and greater than four cysts in 11 cases (31.4 %). Out of these 35 animals, n=33 (94.2 %) originated from MNP, n=1 (2.8 %) from LR, and n=1 (2.8 %) from Berlin.

Suspicious mesocercarial structures encysted in tongue tissues were found in all the samples (n=5) selected for cryotome sectioning. Coincidently, in two raccoons, two similar mesocercarial cysts were found in the adipose tissue adjacent to their thyroid glands.

Mesocercariae identification

In total, 19 mesocercariae were isolated from 11 out of 36 tongues (30.5 %). Figure 2 shows a light microscopy image of an *A. alata* mesocercaria after isolation with AMT. The subsequent PCR analysis of the extracted genomic DNA successfully amplified a region of approximately 300 bp in 13 out of

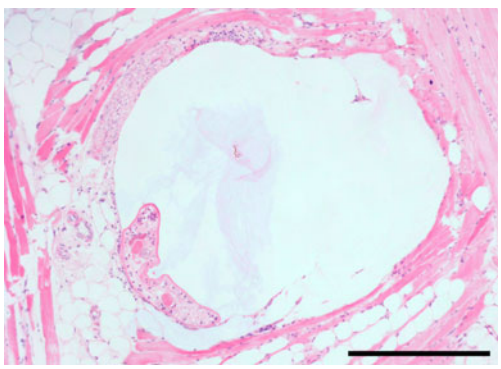

Fig. 1 Raccoon tongue with a parasite cyst containing an intraluminal *A. alata* mesocercaria and a thin basophilic veil. The cyst is surrounded by some lymphocytes and eosinophilic granulocytes and is in close proximity to a small arteriole. *Bar* 500 μm. HE staining

Fig. 2 Light microscopy image of an isolated *A. alata* mesocercaria. *Bar* 200 μm

16 (81.2 %) isolates originating from 11 animals (Fig. 3). Three isolates (18.7 %) produced no band (Fig. 3, lines F, K, and N); two of these were isolated from one single raccoon, and the third isolate originated from a second raccoon which had an additional PCR positive isolate. Table 1 shows the origin and sample site of the isolated mesocercariae and their order in the agarose gel. The positive control, a specimen previously validated as *A. alata* by the National Reference Laboratory for *Trichinella* Diagnostics (German Federal Institute for Risk Assessment), also produced a sharp, single band in the same region. The PCR analysis demonstrated *A. alata* mesocercariae in 10 out of 11 raccoons. Out of these ten positive animals, nine originated from MNP, and one raccoon was collected in the Berlin City center.

Table 1 Origin of isolated mesocercariae used for molecular *A. alata* identification

PCR lane[a]	Mesocercariae from	Raccoon ID	Collecting site
A	Frozen tongue[b]	553/10	MNP[c]
B	Frozen tongue	385/11	Berlin
C	Frozen tongue	218/11	MNP
D	Frozen tongue	346/09	MNP
E	Frozen tongue	48/11	MNP
F	Cryotome section	204/11	MNP
G	Frozen tongue	534/11	MNP
H	Cryotome section	46/11	MNP
I	Cryotome section	204/11	MNP
J	Cryotome section	534/11	MNP
K	Frozen tongue	350/09	MNP
L	Frozen tongue	46/11	MNP
M	Frozen tongue	330/09	MNP
N	Cryotome section	350/09	MNP
O	Cryotome section	46/11	MNP
P	Cryotome section	103/11	MNP

[a] See Fig. 3

[b] Multiple mesocercariae isolated from frozen tongue tissue of a single raccoon were pooled and regarded as a single isolate

[c] Müritz National Park

Discussion

This study reports the detection of *A. alata* mesocercariae in raccoons from Germany. The mesocercarial stage of *A. alata* has been isolated from mustelids like European minks (*Mustela lutreola*), ferrets (*Mustela putorius*) and weasel (*Mustela nivalis*), pine martens (*Martes martes*), sables (*Martes zibellina*), badgers (*M. meles*), as well as from wild boars (*S. scrofa*), moles (*Talpa europaea*), brown bear (*Ursus arctos*), and domestic cattle (Möhl et al. 2009).

In addition to the published parasitological descriptions of isolated *A. alata*, the histological analysis of our investigations allowed a morphological description of *A. alata* mesocercarial cysts as well as possible tissue reactions. It seems characteristic for the mesocercarial cysts to be located within the connective and adipose tissue of the tongues and to contain intraluminal fine basophilic veils. Similarly, in wild boars from Germany, Riehn et al. (2012) found most *A. alata* located in body areas with high amounts of connective and

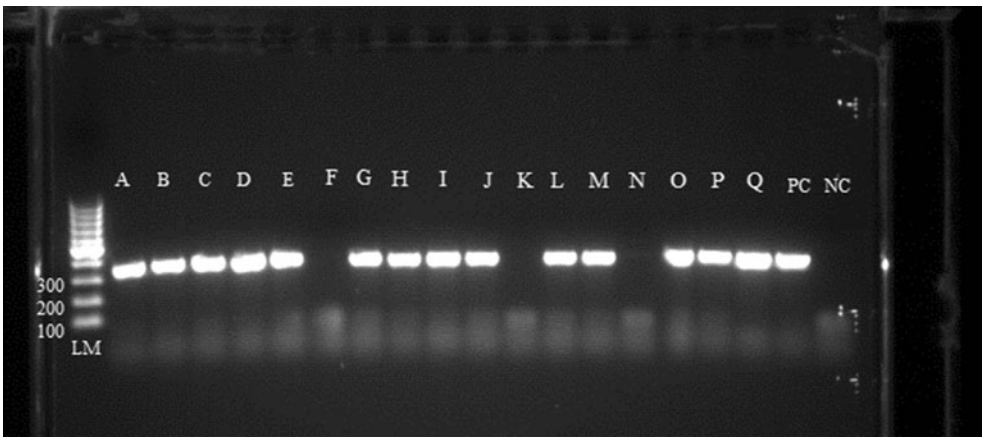

Fig. 3 Agarose gel electrophoresis of *Alaria* spp. PCR products: *lanes A to P Alaria* spp. samples described in Table 1, *lane Q Alaria* spp. collected from wild boar, *LM* DNA marker ladder, *PC* positive control, *NC* negative control

adipose tissues by molecular techniques. Our histological investigations reveal that parasitic cysts are most often located in close proximity to small arterioles, while traces of parasitic migration like necrosis or fibrosis are absent. These findings are indicative for hematogenic spread of the mesocercariae. Known examples of hematogenous parasite spread are *Dirofilaria immitis* or *Sarcocystis neurona*. Iastreb et al. (2005) demonstrated *A. alata* mesocercariae in the blood of domestic dogs and cats.

(Fernandez et al. 1976) reported no inflammatory reaction surrounding *A. americana* mesocercariae in the pulmonary parenchyma of a human patient. Similarly, Shoop and Corkum (1984) described serial histology sections of murine mammary glands with *A. marcianae* mesocercariae migrating through the adipose tissue with notable absence of inflammatory response. The authors indicate that this lack of local immune response might be due to the minimized contact with peripheral leukocytes in adipose tissue (Shoop and Corkum 1984). However, our results show mesocercarial cysts can induce mild local inflammatory response.

Previous investigations indicated that in comparison to other organs routinely investigated, tongue tissues have a high chance of containing the parasite's cysts (unpublished data, G. Wibbelt), which was confirmed in our study. However, the number of cysts found in each tongue does not allow extrapolating to the entire animal's body, while it seems likely that such correlation might be found between different body regions.

Studies related to *A. alata* focus mostly on epidemiological investigations of known hosts, report the isolation from new hosts or describe the improvement of identification methods (Möhl et al. 2009; Riehn et al. 2010, 2011, 2012; Portier et al. 2011). It has also been noticed that the prevalence of *A. alata* in game animals in Germany might be vastly unrecorded (Riehn et al. 2012).

Raccoons should be taken into consideration for further studies regarding *A. alata* epidemiology, ecology, and migration pattern, adding a new survey approach for studies of this parasite in Germany as well as in other European regions.

Conclusions

Previous publications describe *A. alata* mesocercariae in wild boar and other native European animal species. Our results show that *A. alata* can also be isolated from nonnative alien species like the North American raccoon, thus extending the already broad host range of this parasite. The location of mesocercarial cysts in close proximity to small arteries indicates a hematogenous spread through this paratenic host, while the raccoons' tongues seem a suitable and easily accessible sampling site for isolating *A. alata*.

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