Aus dem Institut für Lebensmittelsicherheit und -hygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Alaria alata – Prevalence study in Brandenburg and development of protein- and molecular-based assays for identification of incidental parasite findings during official meat inspection

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Carolyn Marion Kästner
Tierärztin aus Deggendorf

Berlin 2022
Journal-Nr.: 4346

Aus dem Institut für Lebensmittelsicherheit und -hygiene

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Alaria alata –

Prevalence study in Brandenburg and development of protein- and molecular-based assays for identification of incidental parasite findings during official meat inspection

Inaugural-Dissertation

zur Erlangung des Grades eines

Doktors der Veterinärmedizin

an der

Freien Universität Berlin

vorgelegt von

Carolyn Marion Kästner

Tierärztin

aus Deggendorf

Berlin 2022

Journal-Nr.: 4346

Gedruckt mit Genehmigung

des Fachbereichs Veterinärmedizin

der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Uwe Rösler
Erster Gutachter:	Univ.-Prof. Dr. Thomas Alter
Zweiter Gutachter:	Prof. Dr. Karsten Nöckler
Dritter Gutachter:	Prof. Dr. Katharina Riehn

Deskriptoren (nach CAB-Thesaurus):

alaria alata, prevalence, assays, MALDI-TOF, polymerase chain reaction, meat inspection, food safety, meat hygiene, public health, Brandenburg

Tag der Promotion: 06.05.2022

Table of contents

List of abbreviations

A.	*Alaria*
AM	*Alaria alata* mesocercariae
AMT	*Alaria alata* mesocercariae migration technique
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
CHCA	α-cyano-4-hydroxycinnamic acid
COI, *cox I*	Cytochrome c oxidase I
DME	Duncker'scher Muskelegel
dNTPs	Deoxyribonucleotide triphosphates
HrCLM	Hookworm-related cutaneous larva migrans
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
LMS	Larva migrans syndrome
lsrDNA	Large subunit ribosomal RNA gene
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
m/z	Mass-to-charge ratio
OLM	Ocular larva migrans
PCR	Polymerase chain reaction
rRNA	Ribosomal RNA
ssrDNA	Small subunit ribosomal RNA gene
T.	*Trichinella*
To.	*Toxocara*
U.	*Uncinaria*
VLM	Visceral larva migrans

Chapter 1: Introduction

Wild boar meat infested with foodborne parasites can cause human disease due to consumption of raw or insufficiently cooked meat or meat products (Ruiz-Fons 2017, EFSA 2018). One of these parasites is the nematode species *Trichinella* spp. (EFSA 2018). To reduce the risk for consumers of human trichinellosis after oral infection, the European Implementing Regulation (EU) No. 2015/1375 makes official *Trichinella* inspection mandatory for wild boars and other susceptible species intended for human consumption (European Commission 2015). According to Annex I, Chapter I of the Implementing Regulation (EU) No. 2015/1375, official *Trichinella* examination needs to be performed by a method of artificial digestion, while the magnetic stirrer technique is determined as reference detection method (European Commission 2015). In the framework of these examinations, mesocercariae of the trematode *Alaria (A.) alata* as well as nematode larvae other than *Trichinella* spp. appeared as incidental findings in the past few years in Germany and Europe (Möhl et al. 2009, Marucci et al. 2013, Karadjian et al. 2020b).

Amongst the broad spectrum of nematodes other than *Trichinella* spp. found during artificial digestion, non-zoonotic (e.g. *Angiostrongylus vasorum*) and potentially zoonotic helminths (e.g. *Toxocara (To.) cati*, *Ascaris suum*, *Uncinaria (U.) stenocephala*) can be identified (Karadjian et al. 2020b). However, at the beginning of this thesis, a standardized approach for a reliable detection and characterization of these parasites was not available. For this reason, the first aim of this work was to develop a standardized method based on both morphological examination and molecular analysis for a reliable identification of various nematode larvae found during official *Trichinella* inspection.

However, this thesis mainly focusses on *A. alata* mesocercariae (AM), which is a larval stage of the trematode *A. alata* living in the intestine of a definitive host (e.g. fox, dog, wolf) (Odening 1961). *A. alata* has a complex three-host life cycle that can be extended by a paratenic host such as wild boar and potentially humans (Odening 1961). Even though no case reports of human infection with *A. alata* due to consumption of raw or undercooked AM infested wild boar meat exist so far, clinical human disease caused by *A. alata* through consumption of viable AM infested meat cannot be excluded (Odening 1961). For this reason, the Federal Office for the Environment (FOEN) and the Federal Office of Public Health (FOPH) in Bern, Switzerland (Gottstein 2013) as well as the Committee on Biological Agents (ABAS) in Germany (Federal Ministry of Labour and Social Affairs 2020) classified *A. alata* as zoonotic parasite of risk group 2.

To date, data on the prevalence of AM in wild boars from Germany are scarce. Therefore, the second aim of this work was to conduct a prevalence study on *A. alata* in wild boars from the German federal state of Brandenburg to obtain long-term prevalence data and better evaluate temporal and spatial variations in this area over a longer period of time. The federal state of

Brandenburg was selected due to a comparably high AM prevalence determined in this state during the national zoonosis monitoring in 2015 (BVL 2016).

For direct detection and isolation of viable AM from meat samples, the *A. alata* mesocercariae migration technique (AMT) is available (Riehn et al. 2010). Further, an *Alaria* spp.-specific PCR exists (Riehn et al. 2011) to confirm the preliminary diagnosis based on morphological examination. However, molecular methods can be relatively work-intensive, time-consuming and highly costly compared to other detection methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Mayer-Scholl et al. 2016). Further, many diagnostic laboratories do not carry out a large number of different PCR assays to identify a specific microorganism but use MALDI-TOF MS in routine diagnostics.

Therefore, the third aim of this thesis was to develop a standardized MALDI-TOF-based method for reliable identification of AM in wild boar meat. This assay might further be applied by routine laboratories and therefore allows for a comprehensive overview of the AM occurrence in wild boars hunted in different regions of Germany.

Chapter 2: Literature review

2.1 Official *Trichinella* inspection and incidental findings

Official *Trichinella* inspection is mandatory for livestock and game intended for human consumption according to the European Regulation (EU) 2015/1375 (European Commission 2015) and subsequent amendments such as the European Regulations (EU) 2020/1478 (European Commission 2020) and 2021/519 (European Commission 2021). During these examinations, incidental findings such as *Alaria alata* mesocercariae (AM) and larval nematodes different from the *Trichinella* genus are commonly detected (Möhl et al. 2009, Marucci et al. 2013, Karadjian et al. 2020b).

2.1.1 Trichinoscopy

The traditional compressorium technique or trichinoscopy was the only disposable method for *Trichinella* testing before artificial digestion was established in the 1970s (Webster et al. 2006, Riehn et al. 2010). As stipulated in Annex I, Chapter III of the Commission Regulation (EC) 2075/2005, this technique rests on the compression of small pieces of musculature between two thin glasses followed by microscopic investigation (30-40 x magnification or, in case of suspect areas, 80-100 x magnification) for *Trichinella* larvae (European Commission 2005). Concretely, in case of domestic pigs, 28 oat-kernel-size pieces are taken from each diaphragm pillar if both are present. Otherwise, 56 oat-kernel-size pieces are taken from the one existent pillar. In case of wild boars, additionally, seven oat-kernel-size pieces are taken from four defined muscle groups each, giving 28 additional muscle pieces and a total number of 84 pieces (European Commission 2005). However, this method shows a lower sensitivity than digestion methods (Forbes et al. 2003, Ramisz and Balicka-Ramisz 2006, Webster et al. 2006) and does not reliably identify non-encapsulated larvae such as *Trichinella (T.) pseudospiralis* as they do not form a collagen capsule in their nurse cells (Webster et al. 2006, Pozio and Zarlenga 2013). Nevertheless, the compressorium technique was used for AM detection in several studies in the 20th century (Möhl et al. 2009) and beyond (Jakšić et al. 2002).

2.1.2 Artificial digestion

Artificial digestion by magnetic stirrer method (DIN EN ISO 18743:2015) is the reference detection method as stipulated in Annex I, Chapter I of the Commission Implementing Regulation (EU) 2015/1375 (European Commission 2015) in connection with Commission Implementing Regulation (EU) 2020/1478 (European Commission 2020). However, according to Annex I, Chapter II of the Implementing Regulation (EU) No. 2015/1375, further techniques based on artificial digestion are permitted for official *Trichinella* inspection (European Commission 2015). The reference detection method is based on HCl/pepsin digestion of

muscle tissue with a magnetic stirrer and aims for the release of *Trichinella* larvae from meat followed by morphological identification using a stereomicroscope (15-40 x magnification) (Gamble et al. 2000). In preparation for artificial digestion, fat and fascial tissues need to be removed from the samples as they cannot be digested and do not incorporate *Trichinella* larvae (Gamble et al. 2000).

2.2 Helminths other than *Trichinella* found during official meat inspection

A multitude of larval helminths which are not part of the *Trichinella* genus (e.g. *A. alata*, *Toxocara (To.) canis, To. cati, Metastrongylus* spp*., Ascaris suum, Uncinaria (U.) stenocephala*) are commonly found during artificial digestion (Möhl et al. 2009, Marucci et al. 2013, Karadjian et al. 2020b). The mentioned larval nematodes live in different organs and tissues (e.g. liver, lungs, intestinal lumen, blood and lymph vessels) and can falsely be identified as *Trichinella* spp. when infesting the musculature (Marucci et al. 2013). Thus, unlike the adult stage, there is a lack of typical morphological criteria of these larvae, which makes morphological identification extremely difficult and necessitates specialized knowledge in parasitology (Marucci et al. 2013, Karadjian et al. 2020b). Moreover, as assumed by Marucci et al. (2013) and Karadjian et al. (2020b), nematode larvae other than *Trichinella* spp. might become damaged by HCl/pepsin digestion, which makes morphological identification even harder.

2.2.1 Selection of nematodes and pathogenicity

In the following, only a selection of nematodes other than *Trichinella* spp. which are found during official laboratory-based meat inspection is presented. Here, the focus is on *Toxocara* spp. as larvae of this genus are most frequently found during artificial digestion (Marucci et al. 2013, Karadjian et al. 2020b).

The adult stages of *To. canis* and *To. cati* live in the small intestine of canids respectively felids (Glickman and Schantz 1981). Larval nematodes of the *Toxocara* genus are commonly found during official *Trichinella* examination, which is presumably due to their tendency to migrate in non-end hosts (larva migrans) (Marucci et al. 2013). Marucci et al. (2013) detected *To. canis* or *To. cati* in six carnivorous birds, one domestic pig and one badger from Italy during artificial digestion. Further, Karadjian et al. (2020b) found *To. cati* in 14 wild boar samples from France (7), Luxembourg (2) and Germany (5). The frequent occurrence of *Toxocara* spp*.* in these hosts can be explained by the intake of *Toxocara* infested small rodents as well as *Toxocara* eggs contaminating cat or dog faeces (Marucci et al. 2013). The ingestion of *Toxocara* larvae by humans can cause human toxocariasis, which is one of the most frequently reported helminthozoonosis worldwide (Magnaval et al. 2001). Human toxocariasis can vary widely from asymptomatic infections to serious clinical disease, which includes visceral larva migrans

(VLM), ocular larva migrans (OLM), “common” and “covert” toxocariasis as well as neurological toxocariasis (Magnaval et al. 2001, Pawlowski 2001). The VLM syndrome typically occurs in children with contact to puppies at home and is characterized by stomach pains, fever, anorexia, cough, wheeze and hepatomegaly caused by pulmonary and hepatic larval migration (Magnaval et al. 2001). The OLM picture characteristically appears on one side in children and young adults and is marked by vision loss and eye pain connected with several inflammatory eye diseases such as uveitis, endophthalmitis, papillitis or chorioretinitis (Gillespie et al. 1993, Magnaval et al. 2001). While the “common toxocariasis” occurred in adults in the Midi-Pyrenees region of France (Glickman et al. 1987, Magnaval et al. 2001), the “covert toxocariasis” manifests in children (Taylor et al. 1988). Both syndroms are characterized by unspecific clinical symptoms such as stomach pains, fever, dyspnoea, weakness, headache and many more (Magnaval et al. 2001). Also, some authors reported neurological toxocariasis caused by *Toxocara* larvae invading the brain (Wang et al. 1983, Hill et al. 1985, Russegger and Schmutzhard 1989, Rüttinger and Hadidi 1991, Kumar and Kimm 1994, Komiyama et al. 1995, Duprez et al. 1996). Neurological toxocariasis can remain asymptomatic or cause several symptoms such as subtile neurological deficiencies, local or generalized spasms, abnormal behaviour or eosinophilic meningoencephalitis (Pawlowski 2001).

Other helminths quite commonly found by Karadjian et al. (2020b) are *Metastrongylus* spp. and *Ascaris suum*, which both have zoonotic potential (Nejsum et al. 2012, Calvopina et al. 2016). Nematodes of the *Metastrongylus* genus are parasites of the respiratory tract of wild boars and domestic pigs, which are widely spread in wild boars (García-González et al. 2013). These final hosts become infected with *Metastrongylus* spp. by ingestion of earthworms, an intermediate host, carrying the infective larvae (Hobmaier 1929). As an ingestion by earthworms is an essential part of its life cycle and obligatory for its further development, *Metastrongylus* spp. is less prevalent in domestic pigs kept indoor (Hobmaier 1929, García-González et al. 2013). Calvopina et al. (2016) reported a human infection with *Metastrongylus salmi* in the United States, which induced serious respiratory distress, pleural effusion, cough with bloody sputum and exhaustion. However, human infection with *Metastrongylus* spp. might be extremely rare presumably as it is caused by ingestion of earthworms which carry the infective L3 larvae or by mucus secretions of an infested earthworm (Calvopina et al. 2016).

Ascaris suum is commonly found in wild boars and domestic pigs even if kept indoor (Roepstorff and Nansen 1994, De-La-Muela et al. 2001, Popiołek et al. 2010, Castagna et al. 2019). The definitive hosts become infected with *Ascaris suum* by ingestion of faecally excreted infective eggs (Nejsum et al. 2012). The *Ascaris* species infecting humans by ingestion of infective eggs is *Ascaris lumbricoides*, which is closely related to *Ascaris suum* and can hardly be differentiated from the porcine species (Nejsum et al. 2012). Clinical symptoms of human ascariosis included diarrhea, mild hypersensitivity, pneumonia, bloody

sputum and fever (Koino 1922, Phillipson and Race 1967, Lord and Bullock 1982). Despite the host specific nature of *Ascaris* spp., cases of human infection with *Ascaris suum* by ingestion of infective eggs have sporadically been reported (Jaskoski 1961, Phillipson and Race 1967, Crewe and Smith 1971, Roepstorff et al. 2011). In some cases, children were infected with *Ascaris suum* by eating pig manure, which had been dispensed in the garden (Phillipson and Race 1967, Crewe and Smith 1971, Roepstorff et al. 2011). Even though *Ascaris suum* and *Ascaris lumbricoides* both are more suited to their specific host, cross-infections can occur (Koino 1922, Takata 1951, Galvin 1968).

Also, *U. stenocephala* and *Angiostrongylus vasorum* were observed during artificial digestion by Karadjian et al. (2020b). Larvae of hookworms of dogs and cats such as *U. stenocephala* and *Ancylostoma* spp. may induce human skin disease by percutaneous infection, which is known as cutaneous larva migrans (CLM), “creeping eruptions” (Bowman et al. 2010) and – more specifically – as hookworm-related cutaneous larva migrans (HrCLM) (Caumes and Danis 2004). In general, this skin disease is characterized by typical serpiginous, raised, erythematous, pruritic tracks and papules caused by subcutaneously migrating larvae (Davies et al. 1993, Zimmermann et al. 1995, Klose et al. 1996, Blackwell and Vega-Lopez 2001, Beattie and Fleming 2002, Galanti et al. 2002, Diba et al. 2004). HrCLM cases have been reported in different European countries such as Germany, Italy, France, Denmark and the UK (Astrup 1945, Jelinek et al. 1994, Zimmermann et al. 1995, Klose et al. 1996, Blackwell and Vega-Lopez 2001, Galanti et al. 2002, Diba et al. 2004, Herrmann et al. 2004). While most of the registered HrCLM cases can be seen as imported infections from tropical or subtropical countries (e.g. Africa, the Caribbean, South-east Asia, South America) (Davies et al. 1993, Jelinek et al. 1994, Blackwell and Vega-Lopez 2001), some cases reported from Germany and France were considered autochthonous due to extreme weather conditions including high temperatures and humidity (Zimmermann et al. 1995, Klose et al. 1996, Herrmann et al. 2004).

Angiostrongylus vasorum is a metastrongyloid parasite that lives in the right heart and pulmonary vessels of dogs, foxes and several other mammals belonging to the families *Canidae* and *Mustelidae* (Bolt et al. 1994, Koch and Willesen 2009, Morgan and Shaw 2010). Several *Angiostrongylus* species are known to be zoonotic (*Angiostrongylus cantonensis*, *Angiostrongylus costaricensis*) or potentially zoonotic (*Angiostrongylus mackerrasae*, *Angiostrongylus malaysiensis*, *Angiostrongylus siamensis*) (Spratt 2015). In the case of *Angiostrongylus cantonensis*, humans are accidental hosts and might become infected by ingestion of intermediate (e.g. snails, slugs) or paratenic hosts (e.g. prawns, crabs, frogs, lizards) carrying infective L3 larvae. Also, the consumption of vegetables (e.g. lettuce) contaminated with L3 larvae leaving infested snails might be a source of human infection with *Angiostrongylus cantonensis* (Spratt 2015). However, cases of human infection with *Angiostrongylus vasorum* have not been reported yet.

2.2.2 Molecular-based identification

Studies on the molecular identification of nematodes other than *Trichinella* which are detected during official meat inspection are scarce. Marucci et al. (2013) investigated short DNA sequences to identify larval nematodes found during artificial digestion at the species, genus or family level. The authors examined the 18S ribosomal RNA (rRNA) gene, the internal transcribed spacer I (ITS1) gene, the cytochrome c oxidase I (COI) gene and the 12S rRNA gene, which serve as marker genes (Marucci et al. 2013). Using these molecular assays, Marucci et al. (2013) identified 11 of 18 isolates. Concretely, eight isolates were determined at the species level (*To. cati, To. canis*), two at the genus level (*Panagrolaimus* sp., *Metastrongylus* sp.) and one isolate at the superfamily level (*Filarioidea*) (Marucci et al. 2013). Larval nematodes of the remaining seven isolates were not analyzed due to degraded DNA presumably caused by artificial digestion (Marucci et al. 2013). The 18S gene was the preferred marker gene as a PCR product was generated from larvae of all 11 isolates that could be identified (Marucci et al. 2013). However, these 18S PCR products did not allow species identification for the 11 isolates due to structural damage and DNA degradation of some larvae presumably caused by artificial digestion. Therefore, for these isolates, other marker genes such as ITS1, COI and 12S rRNA were needed for species identification (Marucci et al. 2013). Karadjian et al. (2020b) described an approach for the identification of larval nematodes found during artificial digestion which combines both morphological examination and molecular-based assays targeting several genes (18S rRNA, ITS2, *cox I*, 28S rRNA). This work is part of this thesis and will be presented in chapter 4.

2.3 Taxonomy and morphology of *Alaria alata*

The species *A. alata* belongs to the genus *Alaria,* which is part of the family *Diplostomidae*. Its superfamily *Diplostomoidea* belongs to the order *Strigeida* within the subclass *Digenea*. This subclass in turn is part of the class *Trematoda* within the phylum *Platyhelmintha* (Olson et al. 2003, Deplazes et al. 2012).

The adult stage of *A. alata* was described by Goeze (1782) for the first time.

The adult trematode measures about 3-6 x 1-2 mm (Hiepe 1985). Its body is clearly divided into two parts, showing a short, cylindrical posterior body section and a double-sized, leaf-like or cone-shaped anterior body which usually has ear-shaped appendages containing groups of glands (Hiepe 1985). The anterior body section shows an oral sucker, a ventral sucker as well as an elliptical to tongue-shaped tribocytic organ which serves for attachment to the host tissue and extra-intestinal digestion (Hiepe 1985, Lucius et al. 1988, Deplazes et al. 2012). The ventral sucker is smaller than the oral sucker (Hiepe 1985, Lucius et al. 1988). The gonads of the female flukes are situated in pairs in the anterior body section, the lobed testicles of the

male parasites lie one behind the other in the posterior body segment (Hiepe 1985, Lucius et al. 1988, Möhl et al. 2009).
The eggs measure 110-140 x 70-80 µm and are greenish-grey colored (Hiepe 1985, Lucius et al. 1988).
The mesocercarial stage of *A. alata* is known as *Distomum musculorum suis* or Duncker'scher Muskelegel (DME) (Duncker 1896, Odening 1961). *A. alata* mesocercariae (AM) are surrounded by a thin cyst wall and measure 0.4-0.7 x 0.2 mm (Deplazes et al. 2012). They are longitudinally oval shaped, transparent and drawn in a grid pattern. In the anterior body region, there are several head glands whose excretory ducts end at the edge of the oral sucker. A short intestine begins at the oral sucker and ends blindly with horseshoe-shaped legs. In between lies the ventral sucker (Hiepe 1985).
The metacercariae of *Alaria* spp. also measure 0.4-0.7 x 0.2 mm. They have a roundish shape, a thin wall and are nearly transparent (Möhl et al. 2009).

2.4 Life cycle of *Alaria alata*

Alaria spp. have a three-host life cycle. The adult flukes live in the intestine of the definitive hosts and produce eggs. These are excreted with the feces and hatch in water after two weeks, releasing miracidiae, which develop further in a water snail host, e.g. *Planorbis* species (Odening 1961, Hiepe 1985). In this host, the miracidiae reproduce and maturate for approximately one year (reviewed by Möhl et al. (2009)). Then, they actively leave the water snail and enter the second intermediate host in the water. In this second intermediate host (e.g. adult frog, tadpole or another amphibian), the cercariae develop into mesocercariae (Odening 1961). This AM infested second intermediate host can further be ingested by a definitive as well as a paratenic host (Odening 1961). In previous studies, several *Canidae* (e.g. European wolf, dog, red fox, raccoon dog), *Felidae* (e.g. Eurasian lynx, domestic cat) and *Mustelidae* (e.g. stoat, weasel, European otter) were identified as definitive host of *A. alata* in a wide range of countries (Odening 1961, Shimalov et al. 2000, Shimalov and Shimalov 2001, Castro et al. 2009, Al-Sabi et al. 2013, Ozoliņa et al. 2018, Ozoliņa et al. 2020a). After ingestion of an AM infested paratenic or second intermediate host by a definitive host, the mesocercariae go through a somatic migration (Odening 1961). After passing the lungs, they evolve into metacercariae, which migrate via the trachea into the oral cavity of the definitive host. From there, they are swallowed into the small intestine where they develop into adult trematodes and achieve sexual maturity (Odening 1961). The life cycle of *A. alata* is complete when eggs are released from the definitive host's intestine in the environment (Möhl et al. 2009).
By contrast, amphibians, reptiles, birds and mammals such as wild boars, European minks, badgers, cats as well as potentially humans play a role as paratenic hosts in the developmental cycle of *A. alata* (Odening 1961, Deplazes et al. 2012, Tăbăran et al. 2013, Takeuchi-Storm et

al. 2015, Rentería-Solís et al. 2018). Here, larval trematodes go through the intestinal wall into the muscles of the anterior body region and/or settle down in or on several organs having a high affinity to adjacent fat tissue (Odening 1961, Odening 1963, Hiepe 1985, Riehn et al. 2010). In these paratenic hosts, they do not develop any further, but can survive several host changes without any loss of infectivity (Odening 1961, Hiepe 1985).

2.5 Occurrence of *Alaria alata* in Europe

A. alata has already been found in a number of host species (e.g. red fox, wild boar, raccoon, raccoon dog, grey wolf, Eurasian lynx, golden jackal, amphibian) in many European countries such as Germany (Loos-Frank and Zeyhle 1982, Lucius et al. 1988, Riehn et al. 2012, Rentería-Solís et al. 2013, Voelkel et al. 2019), Austria (Duscher 2011, Paulsen et al. 2012, Sailer et al. 2012), Denmark (Al-Sabi et al. 2013, Takeuchi-Storm et al. 2015), Croatia (Jakšić et al. 2002), Hungary (Széll et al. 2013, Berger and Paulsen 2014), Latvia (Ozoliņa et al. 2018, Ozoliņa et al. 2020a, Ozoliņa et al. 2020b, Ozoliņa et al. 2020c), Lithuania (Bružinskaitė-Schmidhalter et al. 2012), Poland (Górski et al. 2006, Bilska-Zając et al. 2020, Strokowska et al. 2020), Czech Republic (Paulsen et al. 2013), Serbia (Ilić et al. 2016, Gavrilović et al. 2019), The Netherlands (Borgsteede 1984), France (Portier et al. 2014, Patrelle et al. 2015), Portugal (Eira et al. 2006), Italy (Gazzonis et al. 2018), Bulgaria (Riehn et al. 2014), Ireland (Wolfe et al. 2001, Murphy et al. 2012) and Belarus (Shimalov and Shimalov 2002). Also, several authors reported findings of *A. alata* in South America, concretely in wild carnivores in Brazil (Ruas et al. 2008, Vieira et al. 2008, Lima et al. 2013) and in domestic cats in Uruguay (Castro et al. 2009). Further, *A. alata* was observed in Corsac foxes in Uzbekistan, Central Asia (Yong et al. 2019) as well as in red foxes in Japan, East Asia (Machida et al. 1975). However, these findings outside Europe are not part of this literature review.

2.5.1 Occurrence of *Alaria alata* in definitive hosts

In several European studies, the prevalence of *A. alata* in red foxes and other wild animals such as raccoon dogs, grey wolves, lynx, otters, American minks and golden jackals was examined. In Germany, the prevalences of adult trematodes found in the intestine of foxes varied widely depending on the richness of lakes and wetlands in the study area. For example, Loos-Frank and Zeyhle (1982) reported an *A. alata* frequency of 0.08% (3/3573) in red foxes in the Southwest of Germany (Baden-Württemberg), while Lucius et al. (1988) showed a prevalence of 29.70% (30/101) in red foxes in the North of Germany (Schleswig-Holstein). Both authors pointed out that the prevalence of *A. alata* is highly associated with a watery area where foxes can ingest AM infested intermediate hosts (Loos-Frank and Zeyhle 1982, Lucius et al. 1988). In other European countries, the prevalence of *A. alata* in red foxes was reported from Austria (0.16% (1/610) in the south, 22.06% (15/68) in the east (Duscher 2011)), Denmark

(34.4%, Al-Sabi et al. (2013)), Hungary (49.2% (413/840) in 2008-2009 and 55.2% (426/772) in 2012-2013 (Széll et al. 2013)), Lithuania (94.8% (255/269) (Bružinskaitė-Schmidhalter et al. 2012)), Latvia (87.4% (471/539) (Ozoliņa et al. 2018)), Poland (13.6% (Górski et al. 2006)), The Netherlands (10.9% (15/137) (Borgsteede 1984)), Portugal (27.4% (Eira et al. 2006)), Ireland (27.3% (Wolfe et al. 2001) as well as 26% and 21% in 2009 and 2010, respectively (Murphy et al. 2012)), Serbia (49.4% (85/172) (Ilić et al. 2016)) and southern Belarus (42.6% (Shimalov and Shimalov 2002)). Further, relatively high *A. alata* prevalences in raccoon dogs were reported from Denmark (69.7% (Al-Sabi et al. 2013)), Lithuania (96.5% (82/85) (Bružinskaitė-Schmidhalter et al. 2012)) and Latvia (83.9% (345/411) (Ozoliņa et al. 2018)). Also, *A. alata* adults were found in wolves in Latvia (92.9% (39/42) (Ozoliņa et al. 2018)) and Poland (26.3% (Górski et al. 2006)) with widely varying prevalences. Moreover, *A. alata* was observed in Eurasian lynx in Latvia (1.7% (4/231) (Ozoliņa et al. 2020a)), in golden jackals in Serbia (30.0% (18/60) (Ilić et al. 2016) as well as in otters (2.6%) and American minks (12.5%) in Poland (Górski et al. 2006).

Further, two studies from Lithuania and Latvia examined the prevalence of *A. alata* metacercariae in red foxes (35.6% (37/104) respectively 10.7% (50/468)) and raccoon dogs (86.8% (45/53) respectively 49.5% (165/333)) (Bružinskaitė-Schmidhalter et al. 2012, Ozoliņa et al. 2018). Additionally, Ozoliņa et al. (2018) detected *A. alata* metacercariae in a grey wolf (2.4% (1/42)) in Latvia.

Also, eggs of *A. alata* were found in the feces from foxes in Ireland (10.1% (Wolfe et al. 2001)) and southern Belarus (55.3% (Shimalov and Shimalov 2002).

2.5.2 Occurrence of *Alaria alata* in intermediate hosts

Only few studies reported the occurrence of AM in second intermediate hosts such as brown frogs and water frogs. Patrelle et al. (2015) investigated the prevalence of AM in water frogs of the *sensu lato* group (e.g. *Pelophylax ridibundus*, *Pelophylax lessonae*, hybrid *Pelophylax esculentus*) and brown frogs of the *sensu lato* group (e.g. *Rana dalmatina*, *Rana temporaria*) in the northeast of France. In this study, the prevalence of AM in brown frogs was 54.1% (53/98), while the prevalence in water frogs was 11.5% (6/52). Further, no significant discrepancies between the AM prevalences of tadpoles and adult frogs were determined in this survey (Patrelle et al. 2015). Also, in the framework of another project, Voelkel et al. (2019) collected water frogs of the *Pelophylax* genus from watery areas around Leipzig, Saxony, Germany and detected AM in 13 of 15 specimens (86.7%). During another study on several amphibians from Latvia, 27.7% (108/390) of all examined animals were tested positive for AM (Ozoliņa et al. 2020c). Here, AM were found in European water frogs (*Pelophylax esculentus* complex) including tadpoles (58.8%; 47/80) and adults (22.4%; 57/255), in adult common frogs (*Rana temporaria*) (15.8%; 3/19) and in adult moor frogs (*Rana arvalis*) (33.3%; 1/3). In

contrast to Patrelle et al. (2015), Ozoliņa et al. (2020c) further showed in their study that within the group of European water frogs, the AM prevalence was significantly higher in tadpoles (58.8%) than in adults (22.4%). The authors assumed that tadpoles might have a higher risk of infection by *A. alata* cercariae because of an increased activity and a thinner skin compared to adult frogs (Ozoliņa et al. 2020c).

2.5.3 Occurrence of *Alaria alata* mesocercariae in paratenic hosts

Several European studies investigated the occurrence of AM in paratenic hosts, especially in wild boars. Since AM are commonly found in the framework of official *Trichinella* inspection of wild boar meat (Möhl et al. 2009), the AM prevalence in wild boars is of major interest with regard to consumer protection. While most studies used the AMT or a modified migration technique (Paulsen et al. 2012, Riehn et al. 2012, Sailer et al. 2012, Paulsen et al. 2013, Rentería-Solís et al. 2013, Berger and Paulsen 2014, Riehn et al. 2014, BVL 2016, Dolle 2016, Gazzonis et al. 2018, Bilska-Zając et al. 2020, Strokowska et al. 2020, Ozoliņa et al. 2020a, Ozoliņa et al. 2020b), other surveys applied artificial digestion (Portier et al. 2014, Gavrilović et al. 2019) or trichinoscopy (Jakšić et al. 2002) for isolation of AM from wild boar meat. The difference in sensitivity of the mentioned isolation techniques, which is described in detail in chapter 2.9.2 of this thesis, should be taken into account when interpreting the following data. In Croatia, an AM occurrence of 1.8% (3/210) was detected in wild boars (Jakšić et al. 2002). In northern Serbia, the presence of AM in wild boars and domestic pigs kept outdoors was examined (Gavrilović et al. 2019). Here, the AM occurrence was 3% (6/200) in wild boars and 2.8% (2/72) in domestic pigs (Gavrilović et al. 2019). In France, Portier et al. (2014) found an AM prevalence of 0.6% (169/27,582) in wild boars in the Rhine valley. In this study, the yearly AM prevalence significantly increased from 1.5% (13/856) in 2007 to 6.6% (36/546) in 2011 (Portier et al. 2014). In two studies, the occurrence of AM in wild boars in the eastern parts of Austria was observed (Paulsen et al. 2012, Sailer et al. 2012). While Paulsen et al. (2012) reported a prevalence of 6.7% (30/451), Sailer et al. (2012) detected AM frequencies of 2.0% (10/490) and 5.5% (4/73). In Hungary, the AM occurrence in wild boars was 1.6% (5/316) (Berger and Paulsen 2014). In Latvia, Ozoliņa et al. (2020b) found an AM prevalence of 43.9% (213/485) in wild boars. Further, highly different data on the AM occurrence in wild boars was reported in two studies from Poland. While Bilska-Zając et al. (2020) determined an AM frequency of 4.2% (151/3589) mainly in the south of the country, Strokowska et al. (2020) showed an AM prevalence of 44.3% (98/221) in the northeast of Poland. As assumed by Strokowska et al. (2020), this high prevalence is associated with the richness of waterbodies in the study area. Also, Bilska-Zając et al. (2020) explained their comparably low AM prevalence by an area of investigation which is rather poor in waterbodies. Paulsen et al. (2013) demonstrated an AM frequency of 6.8% (15/221) in wild boars in southern parts of

Czech Republic which are rich in watery areas. In northern Italy, Gazzonis et al. (2018) observed an AM occurrence of 1% (1/100) in wild boars. In the southeast of Bulgaria, Riehn et al. (2014) found one *A. alata* mesocercaria each in two wild boars. Ozoliņa et al. (2020a) reported the first findings of AM in a Eurasian lynx in Latvia. This fact suggests that the Eurasian lynx might act as both definitive and paratenic host for *A. alata* (Ozoliņa et al. 2020a). Further, Rentería-Solís et al. (2013) detected AM in 11 tongue samples from raccoons in Germany. Here, the authors demonstrated that raccoons can serve as paratenic host for *A. alata* (Rentería-Solís et al. 2013).

In Germany, data on the AM prevalence in wild boars are scarce. In 2015, wild boars were tested for the presence of AM in eight German federal states during a national zoonosis monitoring (BVL 2016). In this study, 4.7 % (45/949) of all sampled wild boars tested positive for AM (BVL 2016). Another survey from East Germany determined an AM prevalence of 3.3% (243/7303) in wild boars in the federal state of Saxony (Dolle 2016).

Further, during a 2-year study from the east of Germany (Brandenburg, Saxony-Anhalt), Riehn et al. (2012) found that 11.5% (33/286) of all tested wild boar samples were AM positive.

2.6 Pathogenicity of *Alaria* spp. and human infections

To date, some cases of human alariosis caused by mesocercariae of *Alaria americana* or not further determined *Alaria* species have been reported (Fernandes et al. 1976, Freeman et al. 1976, McDonald et al. 1994, Kramer et al. 1996). The latter is closely related to *A. alata* and can be found in Canada (Smith 1978) and in the United States (Davidson et al. 1992). McDonald et al. (1994) published two cases of intraocular infection with *Alaria* spp. mesocercariae. Both patients had signs of unilateral diffuse subacute neuroretinitis caused by a mesocercaria of *Alaria* spp. and presumably *A. americana* in the retina and the vitreous, respectively. Both persons were probably infected due to consumption of undercooked infested frog legs in local Asian restaurants in California, United States (McDonald et al. 1994). Another case report described a human infection with mesocercariae leading to pulmonary disease and subcutaneous granuloma on the chest (Kramer et al. 1996). The patient was presumably infected by consumption of rare wild goose meat during a hunting trip in Canada (Kramer et al. 1996). Further, Fernandes et al. (1976) and Freeman et al. (1976) reported a serious generalized human infection with *A. americana* most presumably caused by eating insufficiently cooked frog legs during a hiking trip in Ontario, Canada. This patient developed serious bilateral pneumonia, extensive interstitial hemorrhage and skin petechiae (Fernandes et al. 1976, Freeman et al. 1976). The patient died nine days after presence of the first symptoms because of a pulmonary bleeding which was likely caused by a disseminated intravascular coagulation. During autopsy, several thousands of *A. americana* mesocercariae

were detected in the abdominal cavity and several organs (e.g. lungs, liver, heart, kidneys, brain) (Fernandes et al. 1976, Freeman et al. 1976).

However, no cases of human infection with *A. alata* have been reported so far. Nevertheless, Odening (1961) showed that primates can serve as paratenic hosts for *A. alata*. Here, clinical symptoms were not described (Odening 1961). However, this trematode was recently classified as a zoonotic parasite of risk group 2 by the Federal Office for the Environment (FOEN) and the Federal Office of Public Health (FOPH) in Bern, Switzerland (Gottstein 2013) as well as the Committee on Biological Agents (ABAS) in Germany (Federal Ministry of Labour and Social Affairs 2020). However, using a specific equation based on the AM prevalence in wild boars, the probability that wild boars were not tested and the probability that AM in wild boar meat were inactivated, Ozoliņa et al. (2020b) demonstrated that the probability for humans to become infected with AM through consumption of wild boar meat ranges between 0.2% and 2.2%.

2.7 Tenacity of *Alaria alata* mesocercariae in different matrices

To date, data on the tenacity of AM in different matrices are scarce. As demonstrated by González-Fuentes et al. (2015), AM in Ringer's solution died after storage for 3 minutes at 60.0 ± 2 °C, but they did survive preservation at 4.0 ± 2 °C for up to 13 days.

Further, some studies investigated the survival of AM at freezing temperatures in meat (Hiepe 1985, Portier et al. 2011, González-Fuentes et al. 2015). However, no generally valid statement can be derived from these surveys due to different study designs.

According to Hiepe (1985), AM survive up to eight weeks frozen at – 20 °C in pork. Also, Portier et al. (2011) investigated the cryo-resistance of *A. alata* in wild boar meat. During these investigations, meat was defrosted after a defined number of days of freezing and then examined by artificial digestion (Portier et al. 2011). In this study, Portier et al. (2011) found that AM survive at least five days after freezing at – 18 °C ± 2 °C. After 10 to 19 days post-freezing, AM could no longer be detected using artificial digestion (Portier et al. 2011). However, Riehn et al. (2010) reported that artificial digestion is not suitable for AM detection. As their investigations have shown, AM become highly damaged by the HCl/pepsin digestion followed by a loss of characteristic movement and a high mortality rate (Riehn et al. 2010). For this reason, it cannot be excluded that AM survive significantly longer in frozen wild boar meat, but were simply not detected by artificial digestion as assumed by Portier et al. (2011). Further, González-Fuentes et al. (2015) found that AM in frozen wild boar meat (– 18 °C) did not survive longer than 2 hours when a core temperature of – 13.7 °C was reached. As assumed by González-Fuentes et al. (2015), their different results compared to those by Hiepe (1985) and Portier et al. (2011) might be due to different tissue thickness around the AM. For example,

González-Fuentes et al. (2015) used an average sample weight of 60 ± 0.5 g, while Portier et al. (2011) worked with samples of 100 ± 2 g (González-Fuentes et al. 2015).
Moreover, González-Fuentes et al. (2015) examined the survival of AM in wild boar meat using microwave treatment (power of 8 kW, frequency of 2450 ± 30 MHz). They observed a maximum survival time of 90 seconds with an average internal core temperature of 63.0 ± 0.05 °C in the investigated AM-spiked meat samples (González-Fuentes et al. 2015). However, González-Fuentes et al. (2015) described an uneven heat distribution within the meat pieces as the samples were partially raw in the core, while their surface was predominantly denatured. Due to this fact, the authors presumed that AM nearby the core might survive the microwave treatment (González-Fuentes et al. 2015).
Another survey investigated the tenacity of AM in homemade German meat products such as raw ham, salami and a small raw type sausage known as “Knackwurst” (González-Fuentes et al. 2014). Here, the authors found that no viable AM could be detected in any of the raw hams during or after defined production steps such as curing, equalization, cold smoking and drying. However, viable AM were observed in salami and “Knackwurst” sausages after 24 hours of preparation (fermentation/ripening), but not in the final meat products after the subsequent drying process (González-Fuentes et al. 2014).

2.8 Risk of human exposure to AM infested wild boar meat

In general, it cannot be excluded that AM infested wild boar meat or meat products are placed on the market for human consumption.
However, based on the results of the survival studies mentioned in chapter 2.7, the risk for consumers of human infection with AM through consumption of the examined raw sausage types (salami and “Knackwurst”) (González-Fuentes et al. 2014) infested with AM is considered low (BfR 2017). However, due to a multitude of different raw sausage types, a generally valid statement regarding the risk for consumers by AM infested raw sausage products is currently not possible (BfR 2017). Further, the fact that AM are still viable 24 hours after raw sausage preparation gains in significance when these sausages are consumed directly after production without heating (González-Fuentes et al. 2014). Even though it is recommended to properly cook these sausages before consumption, eating raw sausage products directly after preparation is not unusual among consumers (González-Fuentes et al. 2014). Therefore, special procedure guidelines regarding heating, freezing and handling of raw wild boar meat might be useful to reduce the risk for consumers of human infection by AM infested meat or meat products (González-Fuentes et al. 2014, González-Fuentes et al. 2015). Further, according to Portier et al. (2011), AM infested meat should undergo the same procedure used for inactivation of *Trichinella* spp. larvae, which means heating to a core temperature of 71 °C (Gamble et al. 2000).

2.9 Specific methods for detection of *Alaria alata* mesocercariae

2.9.1 *Alaria alata* mesocercariae migration technique (AMT)

As reported by Riehn et al. (2010), artificial digestion is not an appropriate method for the identification of AM in meat due to a lack of sensitivity. Therefore, the *Alaria alata* mesocercariae migration technique (AMT) was developed as a more sensitive method for detection of AM (Riehn et al. 2010). Briefly, 30 g of the sample material are cut into pieces of about 0.5 cm edge length and carried over to a plastic sieve which is positioned in a glass funnel fixed in a stand (Riehn et al. 2010). Then, the sample pieces are submerged in warm tap water of 46-48 °C for 90 minutes of larval migration and sedimentation. Afterwards, 20 ml of the sedimented sample liquid is investigated in a Petri dish using a trichinoscope or a stereomicroscope (15-20 x magnification) (Riehn et al. 2010). *A. alata* mesocercariae are detected by their characteristic movement and morphological structures (Möhl et al. 2009). According to preliminary results by Riehn et al. (2010), the duration of sedimentation might be shortened from 90 to 30 minutes as more than 95% of the AM were identified within the first 30 minutes of migration (Riehn et al. 2010). These findings were confirmed by Ozoliņa and Deksne (2017) who found that a reduction of the sedimentation time from 90 to 30 minutes is enough to obtain qualitative results for the presence of AM in muscle samples.

2.9.2 Comparison of AMT and artificial digestion for AM detection in meat

Finally, Riehn et al. (2010) showed that the sensitivity of their newly developed AMT to identify AM in wild boar meat is almost 60% higher than that of the official digestion method for *Trichinella* testing. Also, Riehn et al. (2012) found that 11.5% of wild boar samples tested negative for AM during artificial digestion were actually AM positive using AMT. Further, Ozoliņa and Deksne (2017) reported 21.7% of false negative samples by the use of official *Trichinella* inspection method compared to AMT. In addition, Ozoliņa et al. (2020b) observed that the AM prevalence in wild boars was significantly higher using AMT than artificial digestion. These results can be explained by the affinity of AM to fat tissue (Odening 1961, Riehn et al. 2010) which is not examined during artificial digestion. Here, muscles from predilection sites is used from which adipose and connective tissue is removed before testing (Gamble et al. 2000). Moreover, Riehn et al. (2010) demonstrated that AM can die or get damaged by HCl/pepsin digestion, which implies a loss of their characteristic movement and morphological changes. In addition, Ozoliņa and Deksne (2017) assumed that the low sensitivity of artificial digestion for AM detection is due to an inadequate mesh size of the sieves used for the digestion method compared to the size of AM. For this reason, their larger size compared to *Trichinella* larvae might reduce the number of AM caught in the sieves.

2.9.3 Conventional polymerase chain reaction (PCR)

2.9.3.1 General description

Polymerase chain reaction (PCR) is an *in vitro* method that enables the production of millions of copies of certain nucleotide sequences enzymatically (Saiki et al. 1985, Mullis and Faloona 1987, Saiki et al. 1988). Even small amounts of DNA can thus be made accessible for analysis. PCR is performed in a thermal cycler that allows exponential amplification of a defined DNA sequence (Mullis and Faloona 1987, Singh et al. 2014). By detection of a specific gene segment, this method enables molecular identification of a specific microorganism. Concretely, the double stranded target DNA is separated in two single strands (initial denaturation; temperature of 90-95°C for 3-5 minutes). This first step is followed by 20-35 cycles including denaturation, annealing and extension, whereby the number of PCR cycles goes by the concentration of DNA in the complete PCR reaction mix and the expected output of amplified DNA (Saiki et al. 1985, Saiki et al. 1988, Singh et al. 2014). The cyclic repetition of the individual reaction steps allows the initial amount of DNA to be amplified by a factor of about 10^7 (Saiki et al. 1988). After denaturation of the double stranded DNA molecule by heating to 90-95°C (30-55 seconds), the complementary forward and reverse primers bind at the 3' flanking regions of the single stranded DNA template (annealing; temperatures between 50-65°C for 30-55 seconds) (Saiki et al. 1985, Mullis and Faloona 1987, Saiki et al. 1988, Singh et al. 2014). In the next step, the new DNA strand is extended by a thermostable DNA polymerase adding complementary deoxyribonucleotide triphosphates (dNTPs) (extension; temperature of approximately 72°C for 30-55 seconds) (Saiki et al. 1988, Joshi and Deshpande 2010, Singh et al. 2014). As a result, this procedure produces a copy of the initial double-stranded DNA sequence including both an old and a new DNA single strand which both, in turn, can be applied as a template for further copies (Joshi and Deshpande 2010). After the last cycle, the extension temperature of 72°C is held for another 5-15 minutes to complement the overhanging ends of the PCR product (Joshi and Deshpande 2010, Singh et al. 2014). This PCR product consisting of a multitude of copies of the target DNA sequence is finally stored at 4°C in the thermal cycler. In the end, the generated PCR product is visualized as a fluorescent band using agarose gel electrophoresis followed by ultraviolet transillumination of the agarose gel colored by a fluorescence dye (Singh et al. 2014). In the framework of this thesis, the nucleic acid dye GelRed™ was used for staining the agarose gel.

2.9.3.2 PCR targeting *Alaria* spp.

For molecular identification of AM, Riehn et al. (2011) developed a conventional PCR which is based on the amplification of a 303 base pair (bp) region within the complete small subunit ribosomal RNA gene (ssrDNA) and the partial (D1-D3) large subunit ribosomal RNA gene

(lsrDNA) of the *A. alata* genome. These two ribosomal RNA genes were previously applied to estimate the phylogeny of the subclass *Digenea* by Olson et al. (2003).
Further, *A. alata* could recently be identified by Bilska-Zając et al. (2020) using 18S rDNA and cytochrome C oxidase subunit I (COI) gene amplification followed by sequence analysis.

2.9.4 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a standard technique for identification and characterization of bacteria and yeast in diagnostic laboratories (Croxatto et al. 2012, Wieser et al. 2012, Shannon et al. 2018) and has further been applied to virus identification (Calderaro et al. 2014, Calderaro et al. 2016, Iles et al. 2020). Also, this technology has become increasingly popular in parasitology research. For instance, in the field of parasitic helminths, several studies described the application of MALDI-TOF MS for identification of *Trichinella* spp. (Mayer-Scholl et al. 2016, Karadjian et al. 2020a), cyathostomins (Bredtmann et al. 2017), *Anisakis* spp. (Marzano et al. 2020), *Fasciola* spp. (Sy et al. 2021) as well as cercariae of trematodes such as *A. alata* (Huguenin et al. 2019).
Since the MALDI-TOF technique allows a fast characterization of a multitude of microorganisms, it might be applied in diverse fields such as medical diagnostics, environmental monitoring or food quality control (Croxatto et al. 2012).
In preparation of the MALDI-TOF measurements, some microorganisms can be directly spotted onto a target plate without any preliminary treatment (Croxatto et al. 2012). In case of more robust microorganisms (e.g. bacterial spores, yeast, viruses), a preliminary treatment using strong organic acids and/or alcohols is necessary (Croxatto et al. 2012). In a next step, the samples are spotted onto a target plate and blended with a special MALDI matrix, leading to their crystallization within this matrix (Giebel et al. 2010, Croxatto et al. 2012). For MALDI-TOF analysis of proteins, α-cyano-4-hydroxycinnamic acid (CHCA) is known to be an appropriate matrix which is commonly used (Fenselau and Demirev 2001, Croxatto et al. 2012). For this reason, CHCA matrix was selected for the MALDI-TOF project presented in chapter 6 of this thesis.
After inserting the spotted target plate into the mass spectrometer, the mixture of sample and matrix is irradiated by UV laser shots. The laser's energy is absorbed by the matrix, resulting in desorption of the crystallized proteins which are then vaporized and ionized (Giebel et al. 2010, Croxatto et al. 2012, Wieser et al. 2012). In an electric field, the mainly singly charged ions are accelerated in accordance with their mass and electric charge, indicating that smaller ions move faster than larger ones (Giebel et al. 2010, Croxatto et al. 2012, Wieser et al. 2012). These ions fly through a vacuum tube where their time of flight is measured by a detector at

the end of the tube (Giebel et al. 2010, Wieser et al. 2012). This time of flight goes by the mass (*m*) and charge (*z*) of the biomolecule to be analyzed, indicating that molecules with varying m/z are split by their different flying times (Croxatto et al. 2012). The time-of-flight analysis finally allows accurate calculation of the protein mass, resulting in protein spectra characterized by both the mass-to-charge ratio (*m/z*) and the ion intensity representing a fingerprint of any species (Giebel et al. 2010, Croxatto et al. 2012, Wieser et al. 2012). These spectra normally ranging from 1000 to 20 000 *m/z* are compared with those of known isolates stored in a reference spectra database using a specific matching algorithm which allows identification of the unknown microorganism (Demirev et al. 1999, Giebel et al. 2010, Croxatto et al. 2012, Wieser et al. 2012).

Chapter 3: Aims of this thesis

In the past few years, parasites other than *Trichinella* spp. including AM have commonly been found during official *Trichinella* examination using artificial digestion (Möhl et al. 2009, Marucci et al. 2013, Karadjian et al. 2020b). This involves trematodes as well as nematodes.

The broad spectrum of nematode larvae other than *Trichinella* spp. reaches from non-zoonotic (e.g. *Angiostrongylus vasorum*) to potentially zoonotic nematodes (e.g. *To. cati*, *Ascaris suum*, *U. stenocephala*) (Karadjian et al. 2020b). A single method to easily and reliably identify all different nematode species found during *Trichinella* inspection has not been described yet.

Therefore, the first aim presented in this thesis was to develop a morphology- and molecular-based strategy for identification of nematode larvae other than *Trichinella* recovered from muscle samples during mandatory *Trichinella* testing. Even though this method is based on both morphological and molecular inspection, a specialized knowledge of parasitology is not necessarily needed for its application. Further, this standardizable technique enables not only a reliable identification of larval nematodes incidentally found during official *Trichinella* examination, but can also be used to identify isolated nematode larvae in general (Karadjian et al. 2020b).

During the past two decades, AM frequently appeared during official meat inspection of wild boars in many European countries (Möhl et al. 2009). However, data on the prevalence of AM in wild boars from Germany are still rare. Therefore, the second aim of this work was to generate long-term prevalence data on AM in wild boars in the German federal state of Brandenburg and to better estimate the risk for human consumption of wild boar meat infested with viable AM.

To date, AM found during artificial digestion are initially identified by morphological examination using the stereomicroscope. This preliminary diagnosis is currently confirmed by a molecular-based technique (Riehn et al. 2011). However, molecular tools such as the *Alaria* spp.-specific PCR (Riehn et al. 2011) are quite time-consuming, labor-intensive and expensive. By contrast, in recent years, MALDI-TOF-based assays have become increasingly popular in diagnostic laboratories because of their rapidity, simplicity and cost-effectiveness (Wieser et al. 2012).

Therefore, the third and also the main aim of this thesis was to develop a method for a fast and reliable identification of AM in wild boar meat by MALDI-TOF mass spectrometry. Concretely, a protein extraction protocol for one or more AM was established and an AM-specific reference spectra database was created.

In future, this technique should allow all routine laboratories applying MALDI-TOF MS a rapid and reliable identification even of one single *A. alata* mesocercaria.

Chapter 4: A two-step morphology-PCR strategy for the identification of nematode larvae recovered from muscles after artificial digestion at meat inspection

Grégory Karadjian[1], Carolyn Kaestner[2], Lisa Laboutière[1], Emilie Adicéam[1], Tom Wagner[2], Annette Johne[2], Myriam Thomas[1], Bruno Polack[1], Anne Mayer-Scholl[2], Isabelle Vallée[1]

[1] JRU BIPAR, ANSES, INRAE, Animal Health Laboratory, UMR Bipar, École Nationale Vétérinaire d'Alfort,

14 Rue Pierre et Marie Curie, 94701 Maisons-Alfort Cedex, France

[2] Department Biological Safety, German Federal Institute for Risk

Assessment, Berlin, Germany

This chapter has been published in:

Parasitology Research (2020) 119:4113–4122

Received: 15 July 2020

Accepted: 20 September 2020

Published online: 26 September 2020

You have to purchase this part online.

https://doi.org/10.1007/s00436-020-06899-7

Chapter 5: Prevalence of *Alaria alata* mesocercariae in wild boars from Brandenburg, Germany

Carolyn Kästner[1], Nadja Seyhan Bier[1], Anne Mayer-Scholl[1], Karsten Nöckler[1], Martin Heinrich Richter[1] and Annette Johne[1]

[1] Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, 12277 Berlin, Germany

This chapter has been published in:

Parasitology Research (2021) 120:2103–2108

Received: 11 December 2020

Accepted: 29 April 2021

Published online: 8 May 2021

https://doi.org/10.1007/s00436-021-07178-9

Parasitology Research (2021) 120:2103–2108
https://doi.org/10.1007/s00436-021-07178-9

HELMINTHOLOGY - ORIGINAL PAPER

Prevalence of *Alaria alata* mesocercariae in wild boars from Brandenburg, Germany

Carolyn Kästner[1] · Nadja Seyhan Bier[1] · Anne Mayer-Scholl[1] · Karsten Nöckler[1] · Martin Heinrich Richter[1] · Annette Johne[1]

Received: 11 December 2020 / Accepted: 29 April 2021 / Published online: 8 May 2021

Abstract

Since 2002, *Alaria (A.) alata* mesocercariae (AM) have been found during routine *Trichinella* inspection of wild boars in many European countries. To date, human infection with AM through consumption of undercooked or raw AM infested wild boar meat cannot be excluded. In Germany, data on the parasite's prevalence in wild boars are scarce. To better understand temporal and spatial fluctuations of this parasite, this study investigated the prevalence of AM in wild boars in the German federal state of Brandenburg during three hunting seasons from 2017 to 2020. In total, 28.3% (100/354, 95% CI: 23.3–33.3%) of all wild boars sampled in eight counties of Brandenburg were tested positive for AM by *Alaria alata* mesocercariae migration technique (AMT). AM were detected in wild boars from seven different counties. Samples from one county (Havelland) tested completely negative for AM (0/16). Prevalences of the seven AM positive counties of Brandenburg ranged from 11.5 (3/26, 95% CI: 2.5–30.1%) in Märkisch-Oderland to 64.1% (25/39, 95% CI: 47.2–78.8%) in Uckermark. An association between sex and *A. alata* positivity could not be determined. A statistically significant increase in frequency of older AM positive wild boars was observed ($p = 0.001$). For a nationwide assessment of the prevalence of *A. alata* in wild boars and the risk for consumers of ingesting viable AM by consumption of raw or undercooked AM infested wild boar meat, further long-term studies in different regions of Germany are needed.

Keywords *Alaria alata* · Prevalence · Wild boars · Germany · Epidemiology

Introduction

Wild boar meat infested with foodborne parasites can lead to human infection if consumed raw or undercooked (Ruiz-Fons 2017; EFSA 2018). One of these parasites, *Alaria (A.) alata* mesocercariae (AM), are a larval stage of the trematode *A. alata* which have been detected during routine *Trichinella* examination of wild boars in many European countries since 2002 (Möhl et al. 2009; BfR 2017).

A. alata is a parasite which has a complex three-host life cycle. The adult trematodes live in the intestine of several carnivores which serve as definitive hosts (Odening 1961). These adult worms excrete eggs which hatch in water and release miracidiae. These develop further in a water snail host, e.g. *Planorbis* species (Odening 1961; Hiepe 1985). In this host, the miracidiae reproduce and mature for about 1 year (reviewed by Möhl et al. (2009)) before they actively leave the water snail as cercariae and enter the second intermediate hosts in the water. In these second intermediate hosts (e.g. adult frogs, tadpoles or other amphibia), the cercariae develop into mesocercariae (Odening 1961). These AM infested second intermediate hosts can further be ingested by definitive as well as paratenic hosts (Odening 1961). In case of *A. alata*, amphibians, reptiles, birds and mammals such as wild boars, minks, badgers as well as potentially humans rank among paratenic hosts in the developmental cycle (Odening 1961; Deplazes et al. 2012; Takeuchi-Storm et al. 2015; Rentería-Solís et al. 2018). In these hosts, larval trematodes go through the intestinal wall into the muscles of the anterior body region and/or settle down in or on several organs having a high affinity to adjacent fat tissue (Odening 1961; Odening 1963;

Section Editor: Elizabeth Marie Warburton

✉ Annette Johne
annette.johne@bfr.bund.de

Carolyn Kästner
carolyn.kaestner@bfr.bund.de

[1] Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, 12277 Berlin, Germany

Hiepe 1985). In these paratenic hosts, larval trematodes do not develop any further but can survive several host changes without any loss of infectivity (Odening 1961; Hiepe 1985). After ingestion of an AM infected paratenic or second intermediate host by a definitive host, the mesocercariae go through a somatic migration. After passing the lungs, they evolve into metacercariae and later into adult trematodes in the small intestine where they achieve sexual maturity (Odening 1961).

As wild boars are paratenic hosts of *A. alata*, human infection with this trematode through consumption of undercooked or raw wild boar meat or raw meat products cannot be excluded (BfR 2017). Further, *A. alata* has already been classified as zoonotic parasite of risk group 2 by the Federal Office for the Environment (FOEN) and the Federal Office of Public Health (FOPH) in Bern, Switzerland (Gottstein 2013).

The consumption of game meat in Germany has risen over the past decade. According to the German Hunting Association (2019a) which collects data relating to game in Germany, to date, 60% of all Germans consume game meat at least once a year. This amounts to a 25% increase compared to 2009 (German Hunting Association 2019a). The most popular game meat in Germany is wild boar meat and more than 14 tons of wild boar meat were consumed during the hunting season 2018/2019 alone (German Hunting Association 2019a). Traditionally, game meat is consumed fully cooked. However, cooking methods are altering showing a trend towards consumption of inadequately cooked game meat with a pink core as well as raw sausage products (Richomme et al. 2010; Franssen et al. 2017; BfR 2018).

To date, no case reports of human alariosis caused by *A. alata* exist. However, some cases of human infection with *Alaria* spp. respectively *A. americana* mesocercariae have been reported. McDonald et al. (1994) published two cases of intraocular infection with *Alaria* spp. mesocercariae. Both patients probably became infected by consumption of undercooked infested frog legs in local Asian restaurants in California, United States (McDonald et al. 1994). Another case report described a human infection with most likely *Alaria* spp. mesocercariae leading to pulmonary disease and subcutaneous granuloma on the chest (Kramer et al. 1996). The patient was presumably infected through the consumption of raw wild goose meat during a hunting trip in Canada (Kramer et al. 1996). Further, Freeman et al. (1976) reported a serious generalized human infection with *A. americana* after consumption of insufficiently cooked frog legs. The patient developed serious bilateral pneumonia and died of pulmonary bleeding. Also, Odening (1961) demonstrated by experimental infection of a rhesus monkey that primates function as paratenic hosts of *A. alata*. Therefore, clinical disease in humans due to consumption of viable meat or meat products infested with *A. alata* cannot be excluded (Odening 1961).

Article 28 (6) of the Implementing Regulation of the European Commission (EU) 2019/627 (2019) details that carcasses infected with parasites have to be declared unfit for human consumption. Therefore, regionally high *A. alata* prevalences can result in financial losses for hunters and reduce their motivation for hunting (personal communication hunters, expert discussion in October 2016, BfR), increasing e.g. wild boar population densities. This in turn can have detrimental effects on animal disease control and increase crop damage (Massei and Genov 2004).

In 2015, wild boars were tested for the occurrence of *A. alata* in eight German federal states within the framework of the national zoonosis monitoring (BVL 2016). In this study, 4.7% (45/949) of all sampled wild boars tested positive for *A. alata* using the *A. alata* mesocercariae migration technique (AMT) (Riehn et al. 2010). The monitoring also demonstrated regional differences between prevalences in different federal states. For example, the prevalence of Brandenburg was 8.0% (13/163) and therefore almost twice as high as the average prevalence (BVL 2016).

Therefore, the aim of this study was to collect long-term data on the occurrence of *A. alata* in wild boars from Brandenburg to gain a better understanding of temporal and spatial fluctuations in this federal state over time.

Materials and methods

Study design

Using the Epitools Epidemiological Calculator by Sergeant (2018), a required sample size of 114 wild boars was calculated with a confidence level of 95% based on the prevalence of 8.0% determined for the German federal state of Brandenburg during the national zoonosis monitoring in 2015 (BVL 2016). Further, the average annual hunting bag of Brandenburg from 2008/09 to 2018/19 (71708 wild boars (German Hunting Association 2019b)) was used as an estimation of the population size.

The sampling was carried out during hunts organized by the German Federal Forest Service, the Frankenförder Forschungsgesellschaft mbH, and the German Institute for Federal Real Estate (BImA). All animals were legally hunted for human consumption and access to post-mortem sampling was kindly provided by the German Federal Forest Service.

Animal age was defined by the hunters based on teeth eruption, teeth replacement, and physical appearance (Habermehl 1961; Bier et al. 2020). Further, wild boars were divided in three age groups (0, 1, 2). Wild boars younger than 1 year belong to age group 0 and 1 year old animals are part of age group 1. All wild boars aged 2 years or older belong to age group 2 (Game Management Directive of the German federal states of Brandenburg and Mecklenburg-Western Pomerania 2001; Portier et al. 2014; Bier et al. 2020).

Laboratory examinations

After each hunt, the whole tongue and about 30 g of abdominal fat tissue were taken from all sampled wild boars, refrigerated at 4 °C and investigated as a pooled sample with the *A. alata* mesocercariae migration technique (AMT) (Riehn et al. 2010) within a maximum of 7 days after the hunt. Using a stereomicroscope, vital AM were identified by their characteristic morphology and movement and quantified. Further, the parasite load per sample was determined (Table 1).

The morphological identification of the larvae was confirmed by an *Alaria* spp. specific PCR (Riehn et al. 2011). All AM were stored in ethanol absolute at −20 °C. DNA extraction of a single larva was performed using the QIAamp DNA Mini Kit (QIAGEN, 51306) following an adapted Quick-Start Protocol. DNA elution was performed in two consecutive steps with 25 µl of distilled water incubated on the column at room temperature for 3 to 5 min before centrifugation at 8000 rpm in each step. Then, a PCR targeting a 303 bp fragment of the *A. alata* genome was performed as described by Riehn et al. (2011).

Statistical analysis

To assess the effect of sex, age group and hunting season on the prevalence of *A. alata*, the χ^2-test according to Pearson using the software SPSS v. 26 (SPSS Inc., Chicago, IL, USA) was performed. A value of $p \leq 0.05$ was considered as statistically significant. Further, prevalence ratios (PRs) produced by a robust Poisson regression were evaluated to estimate the strength of association (Martinez et al. 2017).

Wilson score intervals were used as confidence intervals for the prevalences. The confidence intervals for the prevalence ratios (PRs) were based on the Poisson regression. Both intervals are confidence intervals of 95%.

Results

In this study, a total of 354 wild boars were sampled during the hunting seasons 2017/2018, 2018/2019 and 2019/2020 in eight counties of the German federal state of Brandenburg. In total, 28.3% (100/354, 95% CI: 23.3–33.3%) of all sampled wild boars in the German federal state of Brandenburg tested positive for *A. alata*. Among all *A. alata* positive wild boars, the number of AM isolated by AMT ranged from zero to 908 AM per sample (pooled tongue and abdominal fat tissue) which resulted in a mean parasite load of 9.60 AM per animal. The mean parasite loads of all AM positive counties reached from 0.23 AM per sample in Märkisch-Oderland to 62.18 AM per sample in Uckermark. AM were detected in wild boars from seven of the eight counties. All samples from county Havelland tested negative for AM (0/16). Prevalences of the seven AM positive counties of Brandenburg ranged from 11.5 (3/26, 95% CI: 2.5–30.1%) in Märkisch-Oderland to 64.1% (25/39, 95% CI: 47.2–78.8%) in Uckermark (Table 1).

In the hunting season 2017/2018, the prevalence was 26.1% (29/111, 95% CI: 18.3–35.3%). In the following hunting season (2018/2019), the prevalence increased to 30.2% (16/53, 95% CI: 18.3–44.3%) and decreased again to 28.9% (55/190, 95% CI: 22.6–36.0%) during the hunting season 2019/2020. Statistically significant differences could not be identified (Table 1; $p = 0.824$).

Further, no association between sex and *A. alata* positivity was determined (Table 1; $p = 0.564$).

However, the prevalence increased with age: 19.6% (29/148, 95% CI: 13.5–26.9%) of all animals of age group 0 were *A. alata* positive. The prevalence rose up to 31.5% (41/130, 95% CI: 23.7–40.3%) in age group 1 and finally to 45.5% (25/55, 95% CI: 32.0–59.5%) in age group 2. The correlation between age and frequency of AM positive wild boars was statistically significant (Table 1; $p = 0.001$). With a PR value of 2.32 (CI: 1.50–3.59; Table 1), wild boars of age group 2 had a prevalence of *A. alata* that was 2.32 times greater than wild boars of age group 0 (Table 1).

Discussion

Wild boar meat is the most popular game meat in Germany (German Hunting Association 2019a) and can pose a risk of human infection with several foodborne parasites (Ruiz-Fons 2017). Due to a lack of knowledge on recent prevalence data of *A. alata* in German wild boars, the aim of this study was to examine the occurrence of *A. alata* in wild boars in the German federal state of Brandenburg over a longer period of time. The federal state of Brandenburg was chosen for this study because of a comparatively high prevalence (8.0%) of *A. alata* during the zoonosis monitoring in 2015.

In this survey, the average prevalence of *A. alata* in wild boars from Brandenburg between 2017 and 2020 was 28.3% (100/354) (Table 1). Compared to some other European prevalence studies of *A. alata* in wild boars, the total prevalence of our study is clearly higher than those found in France (0.6% or 169/27,582) (Portier et al. 2014), Italy (1.0% or 1/100) (Gazzonis et al. 2018), Czech Republic (6.8% or 15/221) (Paulsen et al. 2013), Austria (6.7% or 30/451) (Paulsen et al. 2012) and northern Serbia (3% or 6/200) (Gavrilović et al. 2019). The average prevalence of 4.7% determined in the German national zoonosis monitoring in 2015 (BVL 2016) falls into the mid-range of prevalences mentioned above.

Interestingly, prior studies conducted in the Eastern parts of Germany all showed relatively high prevalences in comparison to other European countries. However, the *A. alata* prevalence of 28.3% detected in our study was nearly 2.5 times

Table 1 Prevalence of *Alaria alata* in wild boars in total and by counties, hunting season, sex, and age group

Variable	Category	No. positive/no. tested animals	Prevalence in % (95% CI)	Range of parasite load (mean parasite load)	*p*-value	PR (95% CI)
Total		100/354	28.25 (23.26–33.25)	0–908 (9.60)		
County	Dahme-Spreewald	29/66	43.94 (31.74–56.70)	0–149 (8.12)		
	Havelland	0/16	0.00 (0.00–20.59)	0		
	Märkisch-Oderland	3/26	11.54 (2.45–30.15)	0–4 (0.23)		
	Oberhavel	13/89	14.61 (8.01–23.68)	0–29 (1.25)		
	Oder-Spree	9/44	20.45 (9.80–35.30)	0–53 (1.98)		
	Ostprignitz-Ruppin	15/53	28.30 (16.79–42.35)	0–67 (2.68)		
	Teltow-Fläming	5/15	33.33 (11.82–61.62)	0–9 (1.07)		
	Uckermark	25/39	64.10 (47.18–78.80)	0–908 (62.18)		
Hunting season	2017/2018	29/111	26.13 (18.25–35.32)		0.824	Reference value
	2018/2019	16/53	30.19 (18.34–44.34)			1.155 (0.690–1.934)
	2019/2020	55/190	28.95 (22.61–35.95)			1.108 (0.755–1.627)
Sex	Male	39/145	26.90 (19.88–34.89)		0.564	Reference value
	Female	56/188	29.79 (23.35–36.87)			1.107 (0.783–1.566)
Age group	0	29/148	19.59 (13.53–26.91)		0.001	Reference value
	1	41/130	31.54 (23.67–40.27)			1.610 (1.065–2.433)
	2	25/55	45.45 (31.97–59.45)			2.320(1.500–3.588)

Note: For statistical analysis, we performed the χ^2 -test according to Pearson. The sex and age group of 21 wild boars as well as the origin (county) of six wild boars were not recorded and could therefore not be used for the analysis

higher than the prevalence of 11.5% demonstrated by Riehn et al. (2012) and even more than 3.5 times higher than the prevalence of 8.0% determined during the national zoonosis monitoring in 2015 (BVL 2016).

Smaller detection rates in other studies might be due to the use of less sensitive methods that were not explicitly developed for AM detection. In the studies from France and northern Serbia for example, all AM were detected by artificial digestion with a magnetic stirrer as used for routine *Trichinella* inspection (Gavrilović et al. 2019). This method is not as sensitive as the AMT described by Riehn et al. (2010) as the mesocercariae can be damaged during digestion (Gavrilović et al. 2019; Portier et al. 2014). According to Riehn et al. (2012), 11.5% (33/286) of all wild boars which tested negative for *A. alata* by artificial digestion were actually AM positive by AMT. Therefore, the true prevalences in the studies in both these countries are presumably higher than determined (Portier et al. 2014). Further, the examination of muscle samples which are not entirely suitable for AM testing could be another reason for smaller detection rates found in other studies. However, in most of the mentioned studies, samples from diaphragm or tongue which both are appropriate tissues (Riehn et al. 2010) were used for AM examination (Riehn et al. 2012; Portier et al. 2014; Gazzonis et al. 2018; Gavrilović et al. 2019). In two studies, mixed samples including diaphragm muscle, glandular and adipose tissue were tested for AM (Paulsen et al. 2012; Paulsen et al. 2013). These sample tissues are also suitable for AM detection due to the affinity of AM to fat, connective and glandular tissue as described by Odening (1961) and Riehn et al. (2010). For this reason, the selection of inappropriate sample material used for AM detection is not an explanation for the lower prevalences found in the studies mentioned above compared to our results.

The current study area, the federal state of Brandenburg, is very rich in lakes, rivers and wetlands (Knittel 2020). This landscape structure offers beneficial conditions for the development and spread of *A. alata* as the parasite's life cycle is associated with intermediate hosts living in or close to surface waters. This could be an explanation for the relatively high prevalence in Brandenburg compared to some of the studies mentioned above.

To find a potential explanation for the wide variation of the prevalence of 28.3% in our study compared to the prevalence of 11.5% determined by Riehn et al. (2012), weather data of Brandenburg provided by the Climate Data Center were analyzed. We focused on the annual average of both the air temperature and the precipitation level from 2007 to 2009 and from 2015 to 2017, the time frames of the two studies.

These were chosen due to the 2-year life cycle of *A. alata*. As reviewed by Möhl et al. (2009), miracidiae maturate for about 1 year in a water snail host before releasing cercariae in the water. Therefore, it should take about 2 years until weather conditions can lead to a noticeable effect on the prevalence of *A. alata* in paratenic hosts such as wild boar.

There were no significant differences in the average temperature during the two studies (10.13 °C vs 9.99 °C) (Climate Data Center 2020a, b).

Between 2007 and 2009, the average annual total precipitation was 663 mm (Climate Data Center 2020c) in comparison to 600 mm between 2015 and 2017 (Climate Data Center 2020d). This variation, however, does not explain the higher prevalence we found in our study indicating that a more in-depth knowledge of both the regional landscape structure as well as the local weather conditions are necessary to understand the size of the parasitic biomass in an area. Further, the higher prevalence in our study compared to the prevalence determined by Riehn et al. (2012) could be justified by an increasing prevalence of finals hosts (e.g. raccoon dogs) in this area. Thus, in the hunting years 2017/18, 2018/19, and 2019/20 which are included in our study, an annual number of 7207, 6572 respectively 6210 raccoon dogs were shot in the federal state of Brandenburg (German Hunting Association 2021). This is a clear rise compared to the hunting years 2009/10 and 2010/11 which were included in the study by Riehn et al. (2012). In these years, an annual number of 5860 respectively 5654 raccoon dogs were shot in Brandenburg (German Hunting Association 2021). Raccoon dogs are known to serve as definitive hosts of *A. alata* (Thiess 2006). Therefore, a growing number of raccoon dogs might be one plausible explanation for the prevalence increase of AM in wild boars in Brandenburg we observed in our survey.

In this study, differences between the prevalences of the eight sampled counties were apparent: While the county Havelland (western part of Brandenburg) had an occurrence of 0.0% (0/16), the prevalence in Dahme-Spreewald (southern part of Brandenburg) was 43.9% (29/66) and in the county Uckermark (northeastern part of Brandenburg) even 64.1% (25/39) (Table 1). These results of a heterogeneous distribution of AM positive wild boars are in line with the findings of the prevalence studies in France (Portier et al. 2014), Austria (Paulsen et al. 2012) and Czech Republic (Paulsen et al. 2013). Due to the small sample size in the county Havelland, the determined occurrence of 0.0% is most probably not representative and needs further verification.

In our survey, a statistically significant correlation between prevalence and age group of the sampled wild boars could be observed. We found a continuous increase of the prevalences of *A. alata* between wild boars of age groups 0 (19.6%), 1 (31.5%) and 2 (45.5%). Similarly, Paulsen et al. (2013) observed a significantly lower prevalence of *A. alata* in wild boars at the age of 1 year and younger (4/22; 18.2%) in comparison to those older than 1 year (11/16; 68.8%). In agreement with Paulsen et al. (2013), it can be assumed that the probability for wild boars to become infected with *A. alata* by eating AM infested prey increases with age.

Similar to the findings in previous studies of Paulsen et al. (2013) and Riehn et al. (2012), a statistically significant association between sex and prevalence of *A. alata* could not be determined.

In conclusion, the regionally very high occurrence of AM in wild boar in Brandenburg, Germany, and the unclear pathogenicity highlight that both further research and discussions on the pathogenicity of the parasite as well as the suitability of cold or heat treatment to kill AM in wild boar meat are needed.

Acknowledgements We thank the German Institute for Federal Real Estate (BImA), the Frankenförder Forschungsgesellschaft mbH and the German Federal Institute for Risk Assessment (BfR)-Center for Land Use Related Evaluation Methods, One Health Approaches for providing assistance and support for this study.

Funding Open Access funding enabled and organized by Projekt DEAL.

References

BfR (2017) Wildschweinfleisch kann den Duncker´schen Muskelegel enthalten. Aktualisierte Stellungnahme Nr. 011/2017 des BfR vom 27. Juni 2017

BfR (2018) Wildfleisch: Gesundheitliche Bewertung von humanpathogenen Parasiten Stellungnahme Nr. 045/2018 des BfR vom 21. Dezember 2018

Bier NS, Stollberg K, Mayer-Scholl A, Johne A, Nöckler K, Richter M (2020) Seroprevalence of Toxoplasma gondii in wild boar and deer in Brandenburg, Germany. Zoonoses Public Health 67(6):601–606

BVL (2016) Zoonosen-Monitoring 2015. Gemeinsamer Bericht des Bundes und der Länder. Berichte zur Lebensmittelsicherheit, BVL-Report 11.2: 30–31

Climate Data Center (2020a) Data retrieved from Climate Data Center (CDC): Jahresmittel der Stationsmessungen der Lufttemperatur auf 2 m Höhe in °C; Version:v19.3 & recent; Abgerufen am 03.11.2020

Climate Data Center (2020b) Data retrieved from Climate Data Center (CDC): Jahresmittel der Stationsmessungen der Lufttemperatur auf 2 m Höhe in °C; Version:v19.3 & recent; Abgerufen am 21.07.2020

Climate Data Center (2020c) Data retrieved from Climate Data Center (CDC): Monatssumme der Stationsmessungen der

Niederschlagshöhe in mm; Version:v19.3 & recent; Abgerufen am 04.11.2020

Climate Data Center (2020d) Data retrieved from Climate Data Center (CDC): Monatssumme der Stationsmessungen der Niederschlagshöhe in mm; Version:v19.3 & recent; Abgerufen am 20.08.2020

Deplazes P, Eckert J, Samson-Himmelstjerna v, Zahner H (2012) Lehrbuch der Parasitologie für die Tiermedizin. 3., überarbeitete Auflage

EFSA (2018) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J 16(12):e05500

Franssen F, Swart A, van der Giessen J, Havelaar A, Takumi K (2017) Parasite to patient: a quantitative risk model for Trichinella spp. in pork and wild boar meat. Int J Food Microbiol 241:262–275

Freeman RS, Stuart PF, Cullen JB, Ritchie AC, Mildon A, Fernandes BJ, Bonin R (1976) Fatal human infection with mesocercariae of the trematode Alaria americana. Am J Trop Med Hyg 25(6):803–807

Game Management Directive of the German federal states of Brandenburg and Mecklenburg-Western Pomerania (2001) Gemeinsame Richtlinie für die Hege und Bejagung des Schalenwildes der Länder Brandenburg und Mecklenburg-Vorpommern (Wildbewirtschaftungsrichtlinie) vom 24. September 2001 (ABl./01, [Nr. 51], S.859). https://bravors.brandenburg.de/de/verwaltungsvorschriften-216896

Gavrilović P, Pavlović I, Todorović I (2019) Alaria alata mesocercariae in domestic pigs and wild boars in South Banat, northern Serbia. Comp Immunol Microbiol Infect Dis 63:142–144

Gazzonis AL, Villa L, Riehn K, Hamedy A, Minazzi S, Olivieri E, Zanzani SA, Manfredi MT (2018) Occurrence of selected zoonotic food-borne parasites and first molecular identification of Alaria alata in wild boars (Sus scrofa) in Italy. Parasitol Res 117(7):2207–2215

German Hunting Association (2019a) Deutschland liebt Wildbret vom Wildschwein" Retrieved from https://www.jagdverband.de/deutschland-liebt-wildbret-vom-wildschwein

German Hunting Association (2019b) Jahresstrecke Schwarzwild (Wildschweine) 2018/2019. Retrieved from https://www.jagdverband.de/jagd-und-wildunfallstatistik

German Hunting Association (2021) Jahresstrecke Marderhund. Data retrieved from https://www.jagdverband.de/sites/default/files/2021-01/2021-01_Infografik_Jahresstrecke_Marderhund_2019_2020.jpg

Gottstein B (2013) Einstufung von Organismen. Modul 3: Parasiten. Stand Januar 2013. Herausgegeben vom Bundesamt für Umwelt BAFU, Bundesamt für Gesundheit BAG, Bern, Schweiz http://www.bafu.admin.ch/uv-1114-d

Habermehl K-H (1961) Altersbestimmung bei Haustieren, Pelztieren und beim jagdbaren Wild: Die Altersbestimmung beim Wildschwein (*Sus scrofa ferus*). Verlag Paul Parey, Berlin und Hamburg, pp. 181–185

Hiepe T (1985) Lehrbuch der Parasitologie Bd 3: Veterinärmedizinische Helminthologie. Gustav Fischer Verlag, Stuttgart

Knittel T (2020) Gewässer in Brandenburg. Data retrieved from https://www.in-berlin-brandenburg.com/Brandenburg/Urlaub/Gewaesser/

Kramer MH, Eberhard ML, Blankenberg TA (1996) Respiratory symptoms and subcutaneous granuloma caused by mesocercariae: a case report. Am J Trop Med Hyg 55(4):447–448

Martinez BAF, Leotti VB, Silva G d S e, Nunes LN, Machado G, Corbellini LG (2017) Odds ratio or prevalence ratio? An overview of reported statistical methods and appropriateness of interpretations in cross-sectional studies with dichotomous outcomes in veterinary medicine. Front Vet Sci 4(193)

Massei G, Genov P (2004) The environmental impact of wild boar. Galemys 16 (n° especial:135–145

McDonald HR, Kazacos KR, Schatz H, Johnson RN (1994) Two cases of intraocular infection with Alaria mesocercaria (Trematoda). Am J Ophthalmol 117(4):447–455

Möhl K, Grosse K, Hamedy A, Wuste T, Kabelitz P, Lucker E (2009) Biology of Alaria spp. and human exposition risk to Alaria mesocercariae—a review. Parasitol Res 105(1):1–15

Odening K (1961) Der Dunckersche Muskelegel kann experimentell auf Affen übertragen werden. Monatshefte für Veterinärmedizin 16: 395–399

Odening K (1963) Zur Diagnostik der Mesocercarie von Alaria alata, eines möglichen Parasiten des Menschen in Europa, an Hand experimenteller Befunde beim Affen. Mber Dtsch Akad Wiss Berlin 5:385–390

Paulsen P, Ehebruster J, Irschik I, Lücker E, Riehn K, Winkelmayer R, Smulders FJM (2012) Findings of Alaria alata mesocercariae in wild boars (Sus scrofa) in eastern Austria. Eur J Wildl Res 58(6):991–995

Paulsen P, Forejtek P, Hutarova Z, Vodnansky M (2013) Alaria alata mesocercariae in wild boar (Sus scrofa, Linnaeus, 1758) in south regions of the Czech Republic. Vet Parasitol 197(1-2):384–387

Portier J, Vallée I, Lacour SA, Martin-Schaller R, Ferté H, Durand B (2014) Increasing circulation of Alaria alata mesocercaria in wild boar populations of the Rhine valley, France, 2007-2011. Vet Parasitol 199(3-4):153–159

Rentería-Solís Z, Kołodziej-Sobocińska M, Riehn K (2018) Alaria spp. mesocercariae in Eurasian badger (Meles meles) and wild boar (Sus scrofa) from the Białowieża Forest, north-eastern Poland. Parasitol Res 117(4):1297–1299

Richomme C, Afonso E, Tolon V, Ducrot C, Halos L, Alliot A, Perret C, Thomas M, Boireau P, Gilot-Fromont E (2010) Seroprevalence and factors associated with Toxoplasma gondii infection in wild boar (Sus scrofa) in a Mediterranean island. Epidemiol Infect 138(9): 1257–1266

Riehn K, Hamedy A, Grosse K, Zeitler L, Lücker E (2010) A novel detection method for Alaria alata mesocercariae in meat. Parasitol Res 107(1):213–220

Riehn K, Hamedy A, Alter T, Lucker E (2011) Development of a PCR approach for differentiation of Alaria spp. mesocercariae. Parasitol Res 108(5):1327–1332

Riehn K, Hamedy A, Grosse K, Wüste T, Lücker E (2012) Alaria alata in wild boars (Sus scrofa, Linnaeus, 1758) in the eastern parts of Germany. Parasitol Res 111(4):1857–1861

Ruiz-Fons F (2017) A review of the current status of relevant zoonotic pathogens in wild swine (S us scrofa) populations: changes modulating the risk of transmission to humans. Transbound Emerg Dis 64(1):68–88

Sergeant E (2018) Epitools epidemiological calculators. Ausvet. Retrieved from https://epitools.ausvet.com.au/oneproportion

Takeuchi-Storm N, Al-Sabi MN, Thamsborg SM, Enemark HL (2015) Alaria alata Mesocercariae among feral cats and badgers, Denmark. Emerg Infect Dis 21(10):1872–1874

Thiess A (2006) Untersuchungen zur Helminthenfauna und zum Vorkommen von Trichinella sp. beim Marderhund (Nyctereutes procyonoides) in Brandenburg. FU Berlin. Digitale Dissertation. Retrieved from https://refubium.fu-berlin.de/handle/fub188/11698

Chapter 6: Development of a novel method for identification of *Alaria alata* mesocercariae by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Carolyn Kästner[1], Peter Bahn[1], Ralph Schönfelder[2], Zanda Ozolina[3], Laura Alksne[3], Martin Heinrich Richter[1], Gunita Deksne[3,4], Anne Mayer-Scholl[1], Annette Johne[1]

[1] Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), 12277 Berlin, Germany

[2] Food Inspection and Veterinary Department, Administrative District Görlitz, 02708 Löbau, Germany

[3] Institute of Food Safety, Animal Health and Environment (BIOR), LV-1076 Riga, Latvia

[4] Faculty of Biology, University of Latvia, LV-1004 Riga, Latvia

This chapter has been published in:

Microorganisms 2021, 9, 1664

Received: 24 June 2021

Accepted: 29 July 2021

Published: 4 August 2021

https://doi.org/10.3390/microorganisms9081664

microorganisms

MDPI

Article

Development of a Novel Method for Identification of *Alaria alata* Mesocercariae by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Carolyn Kästner [1], Peter Bahn [1], Ralph Schönfelder [2], Zanda Ozoliņa [3], Laura Alksne [3], Martin Heinrich Richter [1], Gunita Deksne [3,4], Anne Mayer-Scholl [1] and Annette Johne [1,*]

1 Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), 12277 Berlin, Germany; carolyn.kaestner@bfr.bund.de (C.K.); peter.bahn@bfr.bund.de (P.B.); martin.richter@bfr.bund.de (M.H.R.); Anne.Mayer-Scholl@bfr.bund.de (A.M.-S.)
2 Food Inspection and Veterinary Department, Administrative District Görlitz, 02708 Löbau, Germany; Ralph.Schoenfelder@kreis-gr.de
3 Institute of Food Safety, Animal Health and Environment (BIOR), LV-1076 Riga, Latvia; zanda.ozolina@bior.lv (Z.O.); laura.alksne@bior.lv (L.A.); gunita.deksne@bior.lv (G.D.)
4 Faculty of Biology, University of Latvia, LV-1004 Riga, Latvia
* Correspondence: annette.johne@bfr.bund.de

Citation: Kästner, C.; Bahn, P.; Schönfelder, R.; Ozoliņa, Z.; Alksne, L.; Richter, M.H.; Deksne, G.; Mayer-Scholl, A.; Johne, A. Development of a Novel Method for Identification of *Alaria alata* Mesocercariae by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Microorganisms* **2021**, *9*, 1664. https://doi.org/10.3390/microorganisms9081664

Academic Editor: Chao-Nan Lin

Received: 24 June 2021
Accepted: 29 July 2021
Published: 4 August 2021

Abstract: *Alaria* (*A.*) *alata* mesocercariae (AM) have increasingly appeared as incidental findings during the mandatory inspection of wild boars for *Trichinella* in many European countries. An *Alaria* spp.-specific PCR is available for the identification of AM; however, it is time- and cost-intensive. Therefore, we propose a rapid and cost-efficient MALDI-TOF assay for the identification of AM in wild boar meat that can be applied in routine diagnostics. In this study, a fast and methodologically simple protocol for the protein extraction of AM from different host species in different countries was established, and an AM-specific reference spectra database was created as part of the ongoing development of an existing *Trichinella* spp. database. A formic acid protein extraction was performed after pooling 10 AM from the same host individual. In total, 61 main spectra profiles (MSPs) from different host individuals were stored in an AM-specific MSP library. The cluster analysis of these 61 MSPs indicated a possible variation within the *A. alata* species with a tentative association with the geographical origin of the host, but not the host species. This MALDI-TOF assay allows for a fast verification of the AM isolates, which is the next step in the development of a universal database for the identification of several parasites isolated from meat.

Keywords: *Alaria alata*; MALDI-TOF MS; diagnostics; detection; identification; trematodes; wild boars; foodborne parasitology

1. Introduction

To ensure that meat from livestock or game is safe for human consumption, European legislation lays down rules for mandatory *Trichinella* testing such as Commission Implementing Regulation (EU) No. 2015/1375 [1] and subsequent amendments such as Commission Implementing Regulation (EU) No. 2020/1478 [2]. During the inspection of game meat, wide varieties of parasites, which do not belong to the *Trichinella* genus, are frequently detected [3].

One such parasite is *Alaria* (*A.*) *alata*, whose mesocercariae (AM) have been found with increased frequency in wild boars in Europe during the past few years [4–13].

The adult worms of this parasite live in the intestine of carnivores (e.g., foxes, dogs) and have a complex three-host life cycle that includes wild boars as paratenic hosts [14,15] resulting in possible exposure of humans to the parasite through the consumption of wild boar meat. To date, no human infections caused by the species *A. alata* have been reported. However, Odening [14] demonstrated that primates can function as paratenic hosts for *A.*

alata. In addition, *A. alata* was recently classified as a zoonotic parasite of risk group 2 by the Federal Office for the Environment (FOEN) and the Federal Office of Public Health (FOPH) in Bern, Switzerland [16], as well as the Committee on Biological Agents (ABAS) in Germany [17]. However, based on a specific formula, Ozoliņa et al. [10] demonstrated that the probability for humans becoming infected with AM through consumption of wild boar meat ranges between 0.2% and 2.2%.

To assess the risk of human infection with this parasite and better understand regional and spatial fluctuations of AM in wild boars, several studies on the prevalence of *A. alata* in Germany and other European countries were conducted. In Germany, prevalences between 4.7 and 28.3% were described in different regions [4,11,18]. Further studies were reported from France (0.6%) [7], Italy (1.0%) [13], northern Serbia (3%) [19], Austria (6.7%) [20] and Czech Republic (6.8%) [6]. However, significantly higher prevalences were observed in north-eastern Poland (44.3%) [12] and in Latvia (43.9%) [10].

As the artificial digestion technique, which is the gold standard method for *Trichinella* testing, is not sufficiently sensitive for AM detection in meat, Riehn et al. [21] developed the *A. alata* mesocercariae migration technique (AMT) followed by the morphological identification of AM, which, however, requires a professional expertise in parasitology. Therefore, a confirmation of the AMT results using standardized detection methods, such as molecular or protein-based tools, is absolutely essential for the reliable identification of AM. Molecular methods for AM detection include a specific PCR targeting a 303 base pair (bp) sequence within the complete small subunit ribosomal RNA gene (ssrDNA) and the partial (D1-D3) large subunit ribosomal RNA gene (lsrDNA) of the *A. alata* genome [22] as well as a 18S rDNA and cytochrome C oxidase subunit I (COI) PCR followed by sequence analysis [23]. However, molecular techniques can be relatively work-intensive, time-consuming and expensive [24].

In recent years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has evolved as a routine method for the identification of different microorganisms in many laboratories [25–27]. The advantage of the MALDI-TOF technique is that once a robust, generalized protein extraction protocol and a database incorporating a variety of protein spectra from different microorganisms have been established, there is no need to perform a multitude of different assays to identify the pathogen, resulting in fast and cost-effective identification [25]. In routine diagnostics, MALDI-TOF MS has become a standard tool for identification of bacteria and yeast [25–27] and has also been applied in parasitology research [28–31]. However, in the field of foodborne parasitology, only two studies have demonstrated the use of this technique in the framework of official meat inspection for *Trichinella* spp. [24,32].

Therefore, the aim of this study was to establish a rapid, cost-efficient and methodologically simple protocol for protein extraction of AM and to create an AM-specific main spectra profile (MSP) library as an add-on development to an existing *Trichinella* spp. database.

2. Materials and Methods

2.1. Sample Collection

A total of 61 AM samples from different host individuals were collected during a prevalence study from 2017 to 2020 in Brandenburg, Germany [11], in collaboration with the Food Inspection and Veterinary Department, Administrative District Görlitz (State of Saxony, Germany), the LADR GmbH Medical Care Center North in Flintbek (State of Schleswig-Holstein, Germany), the local veterinary office in Brodnica (Brodnica, Poland) and the Institute of Food Safety, Animal Health and Environment (BIOR) in Riga, Latvia [10,33,34].

All sampled animals were hunted according to each country's hunting regulations, or other permits if necessary.

During the period from 2017 to 2019, amphibians were collected with special permission (26/2017-E; 06.05.2017, 14/2018-E; 10.05.2018 and 21/2019-E-07.05.2019) that was

granted by the Latvian authorities for the collecting and euthanizing of amphibians for scientific purposes (26/2017-E, 14/2018-E, 21/2019-E—Nature Conservation Agency of Latvia).

Adult frogs and tadpoles were gathered from shallow portions using a standard O-frame net with a diameter of 0.6 m, 5-mm mesh size and a handle length of 1.5 m. The collected samples were placed in a disposable box with water (300 mL) and transported to the laboratory within 8 h and kept at +4 °C until further procedures. Euthanasia was performed in the laboratory by a blow to the head as per European Union requirements and the Federation of European Laboratory Animal Science Association regulations (FELASA) [35], under the supervision of a FELASA-certified specialist.

All muscle samples from Saxony, Schleswig-Holstein and Poland were taken from the diaphragm, pharynx or masticatory musculature (Mm. masseter, temporalis, pterygoidei) of wild boars (*Sus scrofa*) that were examined during routine *Trichinella* inspection and transported to the German Federal Institute for Risk Assessment (BfR) in cooling boxes (Table 1). There, the muscle samples were refrigerated at +4 ± 2 °C before being analyzed with the *A. alata* mesocercariae migration technique (AMT) [21] within 24–48 h after arrival in the laboratory.

Table 1. List of *Alaria alata* mesocercariae included in this study. The 8th column of this table gives the log score values for comparison of at least two main spectra profiles created from the same host individual.

No.	Sample Number	Year of Sampling	Host Species	Origin of the Host	Host Tissue Used for AM Isolation	Number of MSPs	Log Scores for MSPs from the Same Host
1	*A. alata* 0573928a	2018	wild boar	Germany, Brandenburg	tongue	2	2.474
2	*A. alata* 0573941	2018	wild boar	Germany, Brandenburg	tongue	1	no value
3	*A. alata* 0573944	2018	wild boar	Germany, Brandenburg	tongue	1	no value
4	*A. alata* 0574243	2018	wild boar	Germany, Brandenburg	tongue	2	2.592–2.593
5	*A. alata* 0662088	2019	wild boar	Germany, Brandenburg	foreleg muscles	1	no value
6	*A. alata* 0667797	2019	wild boar	Germany, Brandenburg	tongue	1	no value
7	*A. alata* 0667798	2019	wild boar	Germany, Brandenburg	tongue	3	2.062–2.599
8	*A. alata* 078901	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.383–2.660
9	*A. alata* 078902	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.316–2.638
10	*A. alata* 078924a	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.011–2.562
11	*A. alata* 078925	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value
12	*A. alata* 078939	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	4	2.308–2.550
13	*A. alata* 078946	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value
14	*A. alata* 078954	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.318–2.623
15	*A. alata* 078957	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	4	2.127–2.430
16	*A. alata* 078966	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value
17	*A. alata* 078987	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.341–2.767
18	*A. alata* 079025a	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	2	2.625–2.632
19	*A. alata* 079028	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	4	2.337–2.647
20	*A. alata* 106797	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.013–2.597
21	*A. alata* 106799	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value

Table 1. *Cont.*

No.	Sample Number	Year of Sampling	Host Species	Origin of the Host	Host Tissue Used for AM Isolation	Number of MSPs	Log Scores for MSPs from the Same Host
22	*A. alata* 170238	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value
23	*A. alata* 193732	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	1	no value
24	*A. alata* 2019030127	2020	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue, diaphragm, chewing muscles	3	2.488–2.681
25	*A. alata* 78953	2019	wild boar	Germany, Brandenburg	tongue, abdominal fat tissue	5	2.108–2.675
26	*A. alata* BZ41794	2019	wild boar	Germany, Saxony	tongue, foreleg muscles	1	no value
27	*A. alata* BZ41796	2019	wild boar	Germany, Saxony	tongue, foreleg muscles	1	no value
28	*A. alata* GOER36711	2018	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	1	no value
29	*A. alata* GOER23024	2019	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	5	2.012–2.491
30	*A. alata* GOER23025	2019	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	5	2.368–2.545
31	*A. alata* GOER32365b	2019	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	3	2.492–2.707
32	*A. alata* GOER32505	2018	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	1	no value
33	*A. alata* GOER33512	2019	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	4	2.468–2.575
34	*A. alata* GOER36908	2019	wild boar	Germany, Saxony	diaphragm, chewing muscles, abdominal fat tissue	5	2.167–2.497
35	*A. alata* GOER481	2018	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	1	no value
36	*A. alata* GOER37662	2019	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	4	2.455–2.619
37	*A. alata* GOER37673	2020	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	4	2.305–2.587
38	*A. alata* GOER37676	2020	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	1	no value
39	*A. alata* GOER44948	2020	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	3	2.668–2.747
40	*A. alata* GOER925	2020	wild boar	Germany, Saxony	diaphragm, stomach, chewing muscles	5	2.000–2.508
41	*A. alata* SH05721106	2019	wild boar	Germany, Schleswig-Holstein	diaphragm	1	no value
42	*A. alata* 1537008	2019	wild boar	Poland, Brodnica	diaphragm	5	2.175–2.579
43	*A. alata* 422-24178	2020	wild boar	Latvia, Alūksnes	diaphragm	1	no value
44	*A. alata* 424-40513	2020	wild boar	Latvia, Balvu	diaphragm	1	no value
45	*A. alata* 420-1-39905	2020	wild boar	Latvia, Burtnieku	diaphragm	2	2.428–2.444
46	*A. alata* 231-88191-2	2018	wild boar	Latvia, Daugavpils	diaphragm, tongue	1	no value
47	*A. alata* 401-31537	2020	wild boar	Latvia, Jaunpiebalgas	diaphragm	1	no value
48	*A. alata* 417-39359	2020	wild boar	Latvia, Jaunpiebalgas	diaphragm	1	no value
49	*A. alata* 11	2020	wild boar	Latvia, Jēkabpils	diaphragm	2	2.520–2.523
50	*A. alata* 252-4404	2019	wild boar	Latvia, Kocēnu	diaphragm, tongue	1	no value
51	*A. alata* 184-72509-2	2018	wild boar	Latvia, Kocēnu	diaphragm	1	no value
52	*A. alata* 408-33448	2020	wild boar	Riga, Limbažu	diaphragm	1	no value
53	A. alata 245-191	2019	wild boar	Latvia, Rēzeknes	diaphragm, tongue	1	no value
54	*A. alata* 418-1-39881	2020	wild boar	Latvia, Rūjienas	diaphragm	9	2.181–2.745
55	*A. alata* 151.3-18	2018	common frog	Latvia, Neretas	serous coat of the internal organs	1	no value

Table 1. *Cont.*

No.	Sample Number	Year of Sampling	Host Species	Origin of the Host	Host Tissue Used for AM Isolation	Number of MSPs	Log Scores for MSPs from the Same Host
56	*A. alata* 152.1-18	2018	common frog	Latvia, Neretas	head	1	no value
57	*A. alata* 10-18	2018	water frog	Latvia, Balvu	serous coat of the internal organs	1	no value
58	*A. alata* 16.7-18	2018	water frog	Latvia, Balvu	inner organs	1	no value
59	*A. alata* 20-18	2018	water frog	Latvia, Balvu	serous coat of the internal organs	1	no value
60	*A. alata* 132.1-18	2018	water frog	Latvia, Dobeles	head	1	no value
61	*A. alata* 238-87828	2018	lynx	Latvia, Siguldas	tongue, different muscles	1	no value

A., *Alaria*; AM, *A. alata* mesocercariae; MSP, main spectrum profile. Note: Sample nos 1–25 came from the prevalence study in Brandenburg, Germany, sample nos 26–42 originating from official *Trichinella* testing were collected by the BfR, Germany, and sample nos 43–61 were gathered by BIOR, Latvia, for research purposes.

During the prevalence study in Brandenburg, whole tongue and about 30 g of abdominal fat tissue were collected from wild boars during hunts, refrigerated at +4 ± 2 °C and transported to the BfR within 3 h.

Prior to examination at the BfR using the AMT [21], all samples were refrigerated at +4 ± 2 °C. The average storage time was 24–48 h, but a few samples were kept for up to 7 days when the number of collected samples could not be processed faster.

Viable AM were detected and provisionally identified based on morphological characteristics, considering the number of glandular cells, body shape, size and movement characteristics using the stereomicroscope at 20× to 100× magnification [15].

All collected AM were stored separately for each host individual in ethanol absolute (Fa. Merck, Darmstadt, Germany) at −20 °C.

All AM from the wild boars, European water frogs (*Pelophylax esculentus* complex), common frogs (*Rana temporaria*) and the Eurasian lynx (*Lynx lynx*) from Latvia were isolated from the diaphragm, tongue, head and inner organs. While AMT [21] was used for the detection of AM in wild boars and the Eurasian lynx, the compression method [36,37] was applied for the isolation of AM from European water frogs and common frogs at BIOR in Riga, Latvia for research purposes. These AM were then transported to the BfR in cooling boxes and stored in ethanol absolute at −20 °C after arrival in our laboratory (Table 1).

In addition to the AM samples, three further samples containing *Opisthioglyphe ranae* larvae from European water frogs from Latvia were provided by BIOR and included in this study to verify that the developed MALDI-TOF technique also allows for a reliable differentiation between trematode species. These samples were also transported to the BfR in cooling boxes and stored in ethanol absolute at −20 °C after arrival.

2.2. Molecular Species Identification of the Samples

For DNA extraction, the QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany, 51306) following an adapted Quick-Start Protocol was used. For the elution step, 25 µL of DNA-free water were used and incubated at room temperature for 3 to 5 min before centrifugation at 8000 rpm. This elution step was repeated once. Apart from the elution step, the protocol was performed according to the manufacturer's instructions.

The DNA extracts from all 61 AM samples were tested by the *Alaria* spp.-specific PCR [22]. All PCR-positive samples were used for the creation of main spectra profiles (MSPs) and stored in the AM-specific MSP library.

Further, the 18S PCR protocol published by Karadjian et al. [3] for the identification of nematodes was applied to 20 randomly selected AM samples, followed by a sequence analysis to determine the suitability of the PCR for the detection and identification of *A. alata*.

In addition, the three samples containing *Opisthioglyphe ranae* larvae were examined using this 18S PCR [3], followed by a sequence analysis, giving a total of 23 larvae-containing samples that were tested by this PCR.

Both the *Alaria* spp.-specific PCR and the 18S PCR were carried out using the protocols published by Riehn et al. [22] and Karadjian et al. [3], respectively.

All PCR products generated by the 18S PCR [3] were sent to Eurofins Genomics (Ebersberg, Germany) for sequence analysis.

2.3. Development of a Protein Extraction Protocol for MALDI-TOF Mass Spectrometry

In general, the AM needed to be fixed in alcohol as handling of the motile larvae was neither practical nor repeatable.

To find a suitable method for protein extraction, several protein sample preparation protocols were tested:

(i) A protocol published by Mayer-Scholl et al. [24] was tested for AM protein extraction but did not result in the generation of reproducible and recognizable protein spectra.

(ii) A single AM was spotted onto the target, followed by air drying, the addition of 70% formic acid and saturated α-cyano-4-hydroxy-cinnamic acid (HCCA). This protocol did not lead to high-quality protein spectra.

(iii) Finally, we developed a working protocol for protein extraction which allows for the generation of main spectra profiles.

To create MSPs, 10 AM isolated from the same host individual and stored in ethanol absolute at −20 °C were used. These AM were washed three times in 96% ethanol and transferred in 10 µL of 96% ethanol in a 0.2 mL Eppendorf tube. The tube was incubated with an open lid in a thermoblock at 50 °C for approximately 30 min or until the liquid had evaporated. The dried AM were visually controlled by light microscopy. For cell disruption, 10 µL of 70% formic acid were added to the dried larvae and mixed by pipetting slowly up and down at least 20 times. Subsequently, the sample was incubated for 10 min at room temperature before spotting onto the target.

Further, the described protocol was optimized for the use of only one single AM using 3 µL of 70% formic acid for cell disruption. All other steps were performed as described above. For optimization, the AM from the same samples as were used for the protocol based on 10 AM were applied.

To verify that the protein extraction protocol based on one single AM is replicable, a total of 38 samples containing one AM each were examined by the BfR. These AM samples came from five different German wild boars (Saxony (2), Brandenburg (3)) and were included in the AM-specific database.

2.4. Generation of an AM-Specific MSP Library

An amount of 1 µL of each protein sample was spotted onto the target plate eight times (MSP 96 target polished steel (MicroScout Target) plate; Bruker Daltonics, Bremen, Germany). For calibration of the MALDI-TOF measurement, 1 µL of Bacterial Test Standard (Bruker Daltonics, Bremen, Germany) was spotted onto the target two times. The air-dried spots were overlaid with 0.8 µL of saturated α-cyano-4-hydroxy-cinnamic acid (HCCA) matrix solution (Bruker Daltonics, Bremen, Germany) and dried completely.

The MALDI-TOF MS measurements were carried out using the MALDI-TOF Microflex LT (Bruker Daltonics, Bremen, Germany) with a range of 2000–20,000 m/z (mass to charge ratio). A total of 24 single spectra per protein sample were acquired from eight spots, whereby each spot was measured three times.

For each single spectrum, 200 laser shots in 40 shot steps from different positions of the target spot (random walk motion) were automatically generated using AutoXecute acquisition mode in FlexControl software (Bruker Daltonics, Bremen, Germany), which was slightly adapted in initial laser power (45%) and maximal laser power (65%). The quality of each spectrum was assessed with FlexAnalysis software (Bruker Daltonics, Bremen,

Germany). The spectra with peak deviations exceeding 500 ppm were not transferred to the MSP library. All high-quality spectra were added to the database using Biotyper 3 software (Bruker Daltonics, Bremen, Germany).

2.5. Evaluation of Generated Protein Spectra by Log Score Values

Log score values are used to measure the reliability of genus and species identification. These values are generated by the comparison of an unknown spectrum with the MSP library as well as the matched peak intensities. The following cutoff scores recommended by Bruker were utilized for sample identification: 0 to 1.699 indicates "no reliable identification"; 1.7 to 1.999 means "probable genus identification"; 2.0 to 2.299 represents "secure genus identification and probable species identification"; and 2.3 to 3.0 indicates a "highly probable species identification".

2.6. Modification of the MSP Identification Method in Biotyper 3 Software

To verify the repeatability of the method, one to nine main spectra from the same host individual were created, depending on the number of AM available, and saved in a MSP database (database 1) (Table 1). However, during these investigations using the protein extraction protocol based on one and 10 AM, slight shifts of the protein spectra of the AM from the same sample on the *x*-axis (mass to charge ratio) were observed. Therefore, the matching of these spectra against one or more already created MSPs from the same sample led to log scores of partially less than 2.0. To solve this problem, the settings of the MSP Identification Method in the Biotyper 3 software were modified as recommended by Bruker (personal communication with the Bruker service department). The aim was that all spectra of AM result in log scores of at least 2.0 compared with the AM-specific MSP library, and that all spectra of *Trichinella* spp. generated by Mayer-Scholl et al. [24] and Karadjian et al. [32] also give log scores of at least 2.0 compared with the *Trichinella* MSP library. Conversely, AM spectra compared with the *Trichinella* MSP library as well as *Trichinella* spp. compared with the AM-specific MSP database should give log scores of less than 1.7. For adaption, single parameters were slightly varied and compared with the resulting score values of the AM and *Trichinella* spp. spectra matched against both the AM- and the *Trichinella*-specific MSP library. Exactly 100 samples of *Trichinella* (*T.*) *spiralis*, *T. pseudospiralis*, *T. britovi*, *T. nativa*, *Hyostrongylus rubidus*, *Trichuris* spp. and an unknown nematode all included in the *Trichinella* database, as well as all AM samples contained in the AM-specific MSP library, were compared with both the AM-specific database and the *Trichinella* MSP library.

Finally, the following settings of the MSP Identification Method were modified: desired mass tolerance of the adjusted spectrum–400 ppm, furthermore accepted mass tolerance of a peak–800 ppm and parameter of the intensity correction function–zero. The remaining parameters (frequency threshold for spectra adjusting, frequency threshold for score calculation and max. mass error of the raw spectrum) were not changed.

2.7. Cluster Analysis

All of the main spectra were compared using MALDI Biotyper 3 software, and their log score values were converted into a cross table in an Excel spreadsheet using a Biotyper Conversion program created by Holger Brendebach (Department of Biological Safety, Federal Institute for Risk Assessment, Berlin, Germany). Based on this cross table, a heat map of the MSPs was created displaying the described cutoff values with different colors (Figure 1). An MSP dendrogram cluster analysis was performed with a correlation distance measurement and single linkage using MALDI Biotyper 3 software (Figure 2). In the MSP dendrogram, a distance level of zero indicates complete similarity and 1000 means complete dissimilarity (Figure 2).

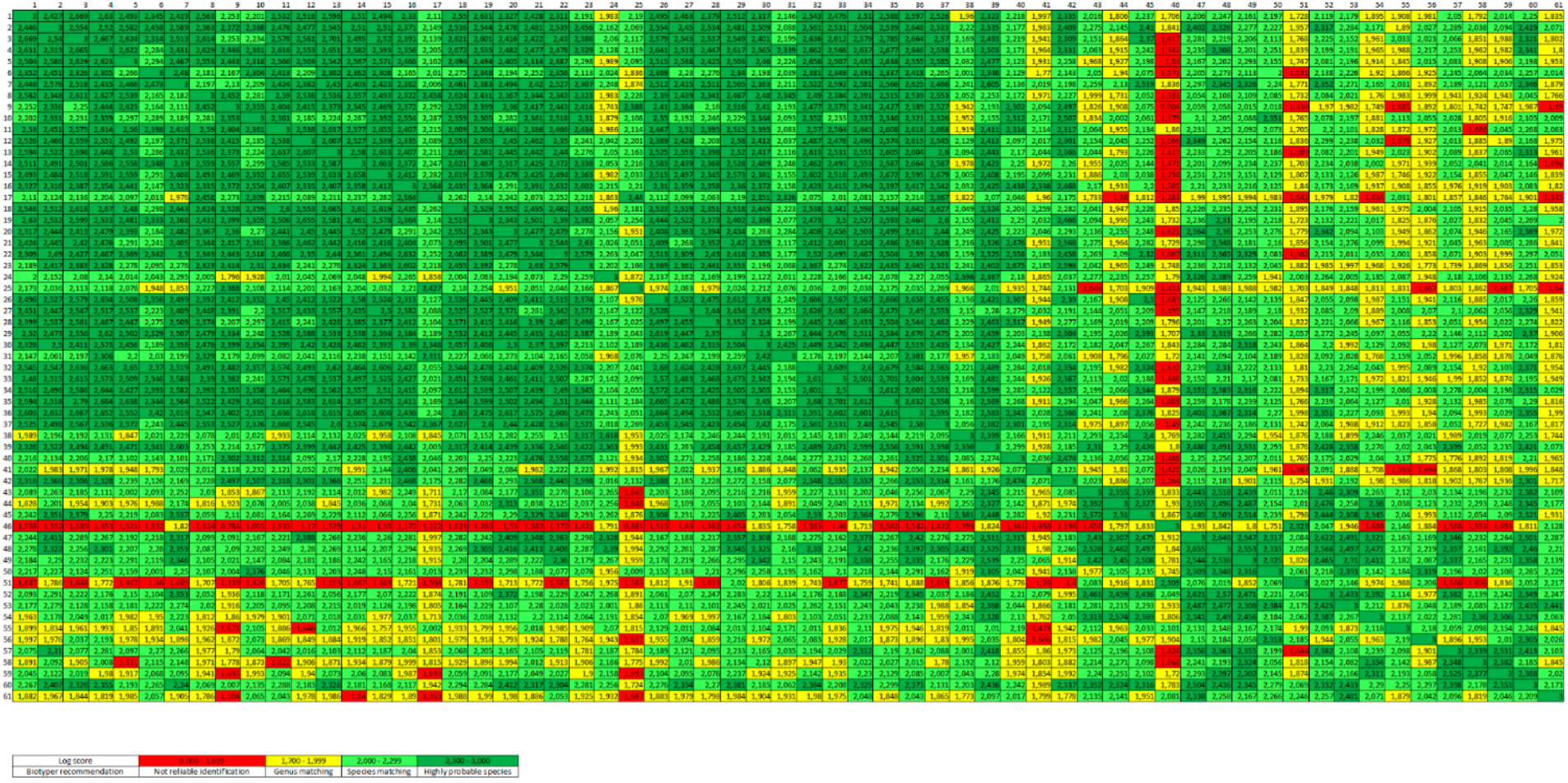

Figure 1. Heat map of 61 *A. alata* main spectra profiles (MSP) based on log scores. Each MSP represents one host individual. The presented figure is sorted by host species, country and region of origin. Nos 1 to 54 are wild boars, nos 55 and 56 are common frogs, nos 57 to 60 are water frogs and no. 61 is a lynx. Further information about the *A. alata* samples are listed in Table 1.

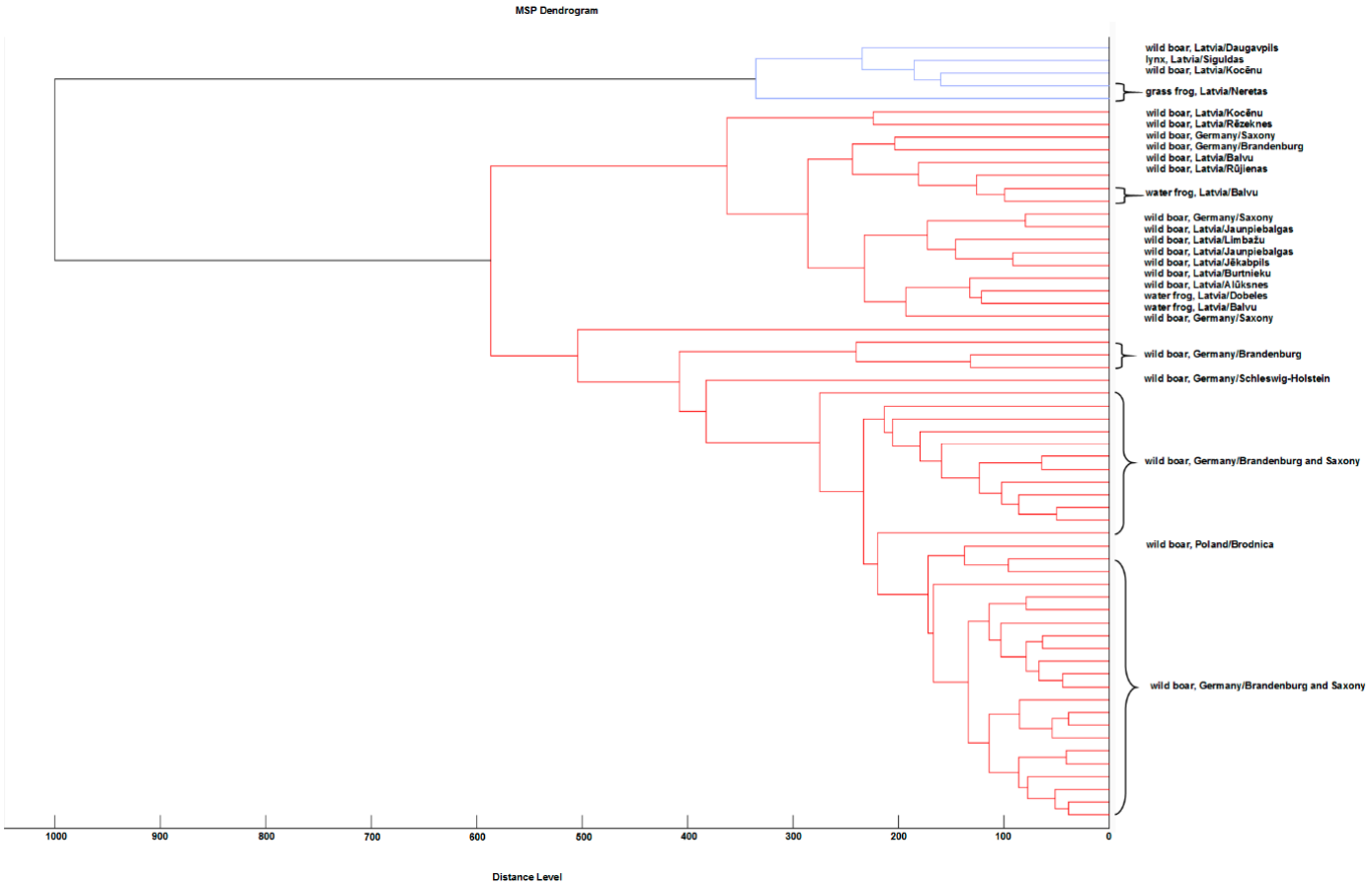

Figure 2. MSP dendrogram cluster analysis of 61 *A. alata* main spectra profiles representing one host individual each.

2.8. Validation of the Developed Protocol Based on 10 AM

To guarantee the reproducibility of our method, the 10 AM protocol was tested in two different laboratories: the BIOR in Riga, Latvia and the BfR in Berlin, Germany. For validation, both laboratories tested seven samples (nos 1–7) containing 10 AM each. AM sample nos 1–3 came from the same wild boar from Saxony, Germany; AM samples nos 4–7 were from three different wild boars from Latvia and sample nos 4 and 5 came from the same wild boar. All wild boars from Germany and Latvia had already been included in the AM-specific database.

3. Results

3.1. Origin of the Samples

In total, 61 AM samples were identified morphologically as *A. alata* using the stereomicroscope. For all samples, these results were confirmed by the *Alaria* spp.-specific PCR [22].

The 61 AM positive muscle samples came from naturally AM infected wild boars (n = 54), European water frogs (n = 4), common frogs (n = 2) and a Eurasian lynx (n = 1). 41 wild boars originated from Germany (Brandenburg (25/41), Saxony (15/41) and Schleswig-Holstein (1/41)), one wild boar came from Poland (Brodnica). 19 samples originated from different regions in Latvia and were isolated from wild boars (12/19), European water frogs (4/19), common frogs (2/19) and a Eurasian lynx (1/19) (Table 1).

3.2. Molecular Analysis of the Samples

From the 61 *Alaria*-specific PCR positive samples, 20 were further confirmed as *A. alata* by 18S PCR [3] followed by sequencing. In addition, the three samples containing *Opisthioglyphe ranae* larvae were examined by 18S PCR [3] and identified as such (99% identity each).

Interestingly, the 18S sequencing protocol by Karadjian et al. [3] described for identification of nematodes showed complete correlation for the detection of trematodes. However, in all examinations, the fragment length of the PCR products from trematodes was about 750 bp and therefore 100 bp larger than the fragment length of the nematodes described by Karadjian et al. [3] (650 bp).

3.3. Creation of Main Spectra Profiles (MSPs)

From all 61 AM samples, a total of 148 main spectra were initially generated using this newly developed MALDI-TOF technique (database 1) (Table 1). After the modification of the MSP Identification Method, 61 of the original 148 main spectra remained. These 61 MSPs represent one host individual each and were included in the newly established AM-specific reference spectra database (database 2).

3.4. Cluster Analysis of the Created MSPs

For a cluster analysis of these AM main spectra, a heat map was created where two clusters with different regional origins of AM were observed (Figure 1). The large German cluster contained all wild boar spectra from Germany (nos 1–40) and Poland (no. 42), which showed mainly log scores of at least 2.0. Within this cluster, three main spectra from wild boars from Brandenburg (no. 24 and no. 25) and Saxony (no. 38) demonstrated slight differences (some with log scores between 1.7 to 1.9) (Table 1, Figure 1). In addition, the main spectrum from the German federal state of Schleswig-Holstein (no. 41) presented many score values of 1.7 to 1.9 when compared with the rest of the German cluster, and therefore clearly differed from this cluster. The small Latvian cluster was formed by all the Latvian main spectra from the four different host species: wild boar, common frog, European water frog and Eurasian lynx (nos 43–61). However, two wild boar spectra (no. 46 and no. 51) showed major differences (log scores of 0.7 to 1.9) and were therefore excluded from this cluster. Apart from these two main spectra (no. 46 and no. 51), most wild boar spectra from Latvia were approximately in agreement with the results of the German cluster (nos 43, 45, 47, 48, 49, 50, 52 and 53) or showed only slight variations (no. 44 and no. 54) (Figure 1).

The results described in the heat map are nearly mirrored in the MSP dendrogram depicted in Figure 2. Here, the two clusters can be seen more prominently. The small cluster contained five Latvian spectra from wild boars (no. 46 and no. 51), common frogs (no. 55 and no. 56) and the Eurasian lynx (no. 61) (Figure 2). The big cluster contained two subclusters including 17 (cluster 1), respectively 39 (cluster 2) main spectra. In difference to Figure 1, the first subcluster included both Latvian (wild boars (10), European water frogs (4)) and German (wild boars (3), nos 24, 38 and 39) main spectra, while the second subcluster was formed only by wild boar main spectra from Germany (Saxony, Brandenburg and Schleswig-Holstein) (38) and Poland (Brodnica) (1) (Figure 2).

3.5. Sensitivity, Reproducibility and Repeatability of this MALDI-TOF Technique

Further, the three *Opisthioglyphe ranae* samples tested negative for AM using MALDI-TOF MS (log scores less than 1.7), demonstrating that this method also allows a reliable differentiation between trematode species.

The reproducibility of the protocol based on 10 AM was shown as six of seven samples tested in the two separate laboratories showed log score values in the range of 2.2–2.5, and one sample gave values from 1.8 to 2.4.

The repeatability of the one AM protocol was demonstrated by the BfR as 36 of 38 AM showed log scores of 2.0 and more in all three single spectra. Two AM each gave two single

spectra with score values of at least 2.0; one spectrum each showed genus matching (log scores of 1876, respectively 1966).

4. Discussion

4.1. Background and Context to Previous Studies

Due to the high frequency of incidental AM findings in wild boar carcasses and the fact that there is no mandatory AM testing of wild boars to date, an exposure of humans to *A. alata* via the consumption of wild boar meat cannot be excluded.

The presence of AM in second intermediate and paratenic hosts previously reported in several European countries were all based on the morphological identification of AM and/or molecular methods [4,6,7,10–13,18–20]. Furthermore, DNA extraction protocols have been established and several different primer pairs developed to examine the genetic diversity of *A. alata* [22,23,38,39]. By contrast, this study presents MALDI-TOF MS as a rapid, cost-efficient technique to be used as a standard tool and a future trend for the identification of *A. alata*.

To our knowledge, the only other study using MALDI-TOF MS for the identification of *A. alata* is presented by Huguenin et al. [31]. In contrast to our study, the authors showed low intra-species heterogeneity when visualizing *A. alata* cercariae from various snail species from France in a MSP dendrogram (distance level < 100). A similar protein extraction protocol was implemented which only differed in the addition order of the same reagents [31]. However, the protein spectra of *A. alata* cercariae generated in this study did not match with the protein spectra of AM created in our study. This may be due to the slightly differing protein extraction protocols or the different host species. Nevertheless, the varying protein patterns are most likely associated with the different developmental stages of *A. alata* (cercariae vs. mesocercariae), which may be examined in further studies.

4.2. Interpretation of the Cluster Analysis

In the present survey, the cluster information obtained through the analysis of 61 MSPs from different host animals indicated possible variation within the *A. alata* species, with a tentative association with the geographical origin of the host, but not the host species. The AM sample from Poland, which is the neighboring region of East Germany, clusters with the East-German samples. By contrast, the Latvian AM samples originating from North-Eastern Europe formed their own cluster, even if some wild boar spectra and one water frog spectrum also fit in the above-mentioned German/Polish cluster. A total of 12 spectra (11 from the North-Eastern and one from the German/Polish group) did not cluster according to geographical origin. At this point, the possibility of identifying intra-species variability is purely speculative due to the small size of the samples in this study. Nevertheless, Bilska-Zając et al. [23] recently published a manuscript describing intraspecific genetic variability among AM specimens, i.e., 17 different genotypes of AM. However, in this study, a direct association between the genotype of this parasite and the host's geographical origin was not observed [23].

4.3. Future Potential of the MALDI-TOF Technique

Currently, the identification of zoonotic or potentially zoonotic parasitic species other than *Trichinella* spp. is fully dependent on either morphological classification [15] and/or PCR [22] or even PCR followed by sequencing [3,40], making this procedure unsuitable for use in routine laboratories. As the MALDI-TOF technique has been implemented in many routine diagnostic laboratories in past years, a generally available protocol for the analysis of parasitic pathogens and contaminants would be advantageous. The specificity of the MALDI-TOF method is fully sufficient to distinguish between the genus *Trichinella* and *Alaria* (log score values < 1.700, data not shown), and even has the potential to distinguish between *Trichinella* species, or perhaps even between genotypes [24].

In this study, a future trend of MALDI-TOF MS is presented as a standard tool for the identification of *A. alata*, including the capability of differentiation between trematode

species. Currently, a protocol is under development to harmonize the protein extraction for both nematodes and trematodes isolated from meat.

5. Conclusions

The aim of this study was to develop a standardized MALDI-TOF assay for the rapid and reliable identification of AM in wild boar meat. Protein extraction protocols based on one and 10 AM were established and pre-validated. Furthermore, an AM-specific reference spectra database including 61 MSPs from different host individuals was created.

The long-term objective is to develop a unique protein extraction protocol and to generate a universal database for the identification of several parasites (e.g., *Trichinella* spp., *Toxocara canis/cati*, *Ascaris suum*, *Metastrongylus* spp. and *Uncinaria stenocephala*) isolated from meat.

Author Contributions: Conceptualization, C.K., A.J. and A.M.-S.; investigation, C.K., P.B., Z.O. and L.A.; resources, C.K., A.J., R.S., G.D., Z.O. and L.A.; data curation, C.K., A.J., Z.O., L.A. and G.D.; writing—original draft preparation, C.K.; writing—review and editing, A.J., A.M.-S., P.B., M.H.R., G.D., Z.O., L.A. and R.S.; visualization, C.K. and P.B.; supervision, A.J. and A.M.-S.; project administration, A.J. and A.M.-S.; funding acquisition, A.J., R.S., G.D. and M.H.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The LADR GmbH Medical Care Center North in Flintbek, Germany and the local veterinary office in Brodnica, Poland are greatly acknowledged for the provision of muscle samples. We especially thank all employees of the Food Inspection and Veterinary Department, Administrative District Görlitz, Germany, in particular Werner Weszkalnys for providing numerous sample materials and support for this project. Further, Muza Kirjušina (Daugavpils University, Latvia) is greatly acknowledged for providing trematode larvae samples from frogs.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. European Commission. Commission Implementing Regulation (EU) 2015/1375 of 10 August 2015 Laying down Specific Rules on Official Controls for Trichinella in Meat. *Off. J. Eur. Union* **2015**, *58*, 7–34.
2. European Commission. Commission Implementing Regulation (EU) 2020/1478 of 14 October 2020 amending Implementing Regulation (EU) 2015/1375 as regards sampling, the reference method for detection and import conditions related to *Trichinella* control C.I.R. *Off. J. Eur. Union* **2020**, *3*, 1–3.
3. Karadjian, G.; Kaestner, C.; Laboutière, L.; Adicéam, E.; Wagner, T.; Johne, A.; Thomas, M.; Polack, B.; Mayer-Scholl, A.; Vallée, I. A two-step morphology-PCR strategy for the identification of nematode larvae recovered from muscles after artificial digestion at meat inspection. *Parasitol. Res.* **2020**, *119*, 4113–4122. [CrossRef] [PubMed]
4. Riehn, K.; Hamedy, A.; Grosse, K.; Wüste, T.; Lücker, E. *Alaria alata* in wild boars (*Sus scrofa*, Linnaeus, 1758) in the eastern parts of Germany. *Parasitol. Res.* **2012**, *111*, 1857–1861. [CrossRef]
5. Sailer, A.; Glawischnig, W.; Irschik, I.; Lücker, E.; Riehn, K.; Paulsen, P. Findings of *Alaria alata* mesocercariae in wild boar in Austria: Current knowledge, identification of risk factors and discussion of risk management options. *Wien. Tierärztliche Mon.* **2012**, *99*, 346–352.
6. Paulsen, P.; Forejtek, P.; Hutarova, Z.; Vodnansky, M. *Alaria alata* mesocercariae in wild boar (*Sus scrofa*, Linnaeus, 1758) in south regions of the Czech Republic. *Vet. Parasitol.* **2013**, *197*, 384–387. [CrossRef]
7. Portier, J.; Vallée, I.; Lacour, S.A.; Martin-Schaller, R.; Ferté, H.; Durand, B. Increasing circulation of *Alaria alata* mesocercaria in wild boar populations of the Rhine valley, France, 2007–2011. *Vet. Parasitol.* **2014**, *199*, 153–159. [CrossRef]
8. Riehn, K.; Lalkovski, N.; Hamedy, A.; Lücker, E. First detection of *Alaria alata* mesocercariae in wild boars (*Sus scrofa* Linnaeus, 1758) from Bulgaria. *J. Helminthol.* **2014**, *88*, 247–249. [CrossRef]
9. Maleševic, M.; Smulders, F.J.M.; Petrovic, J.; Mirceta, J.; Paulsen, P. *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) in northern Serbia after the flood disaster of 2014. *Wien. Tierarztl. Mon.* **2016**, *103*, 345–349.
10. Ozoliņa, Z.; Mateusa, M.; Šuksta, L.; Liepiņa, L.; Deksne, G. The wild boar (*Sus scrofa*, Linnaeus1758) as an important reservoir host for *Alaria alata* in the Baltic region and potential risk of infection in humans. *Vet. Parasitol. Reg. Stud. Rep.* **2020**, *22*, 100485.

11. Kästner, C.; Bier, N.S.; Mayer-Scholl, A.; Nöckler, K.; Richter, M.H.; Johne, A. Prevalence of *Alaria alata* mesocercariae in wild boars from Brandenburg, Germany. *Parasitol. Res.* **2021**, *120*, 2103–2108. [CrossRef]
12. Strokowska, N.; Nowicki, M.; Klich, D.; Bełkot, Z.; Wiśniewski, J.; Didkowska, A.; Chyla, P.; Anusz, K. The occurrence of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) in north-eastern Poland. *Int. J. Parasitol. Parasites Wildl.* **2020**, *12*, 25–28. [CrossRef]
13. Gazzonis, A.L.; Villa, L.; Riehn, K.; Hamedy, A.; Minazzi, S.; Olivieri, E.; Zanzani, S.A.; Manfredi, M.T. Occurrence of selected zoonotic food-borne parasites and first molecular identification of *Alaria alata* in wild boars (*Sus scrofa*) in Italy. *Parasitol. Res.* **2018**, *117*, 2207–2215. [CrossRef]
14. Odening, K. Der "Dunckersche Muskelegel" kann experimentell auf Affen übertragen werden. *Mon. Für Veterinärmedizin* **1961**, *16*, 395–399.
15. Möhl, K.; Große, K.; Hamedy, A.; Wüste, T.; Kabelitz, P.; Lücker, E. Biology of *Alaria* spp. and human exposition risk to *Alaria* mesocercariae-a review. *Parasitol. Res.* **2009**, *105*, 1–15. [CrossRef]
16. Gottstein, B. Einstufung von Organismen. Modul 3: Parasiten. Stand Januar 2013. Herausgegeben vom Bundesamt für Umwelt BAFU, Bundesamt für Gesundheit. BAG: Bern, Switzerland, 2013. Available online: http://www.bafu.admin.ch/uv-1114-d (accessed on 19 February 2021).
17. Federal Ministry of Labour and Social Affairs, Technische Regeln für Biologische Arbeitsstoffe (TRBA) 464 "Einstufung von Parasiten in Risikogruppen", Ausgabe Juli 2013, 1. Änderung vom 10.11.2020, GMBI Nr. 45. 2020. Available online: https://www.baua.de/DE/Angebote/Rechtstexte-und-Technische-Regeln/Regelwerk/TRBA/pdf/TRBA464.pdf?__blob=publicationFile&v=3 (accessed on 26 April 2021).
18. BVL, Zoonosen-Monitoring 2015. Gemeinsamer Bericht des Bundes und der Länder. Berichte zur Lebensmittelsicherheit, BVL-Report 11.2: 30-31. 2016. Available online: https://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/04_Zoonosen_Monitoring/Zoonosen_Monitoring_Bericht_2015.pdf%3F__blob%3DpublicationFile%26v%3D6 (accessed on 24 August 2020).
19. Gavrilović, P.; Pavlović, I.; Todorović, I. *Alaria alata* mesocercariae in domestic pigs and wild boars in South Banat, northern Serbia. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *63*, 142–144. [CrossRef]
20. Paulsen, P.; Ehebruster, J.; Irschik, I.; Lücker, E.; Riehn, K.; Winkelmayer, R.; Smulders, F.J.M. Findings of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) in eastern Austria. *Eur. J. Wildl. Res.* **2012**, *58*, 991–995. [CrossRef]
21. Riehn, K.; Hamedy, A.; Große, K.; Zeitler, L.; Lücker, E. A novel detection method for *Alaria alata* mesocercariae in meat. *Parasitol. Res.* **2010**, *107*, 213–220. [CrossRef] [PubMed]
22. Riehn, K.; Hamedy, A.; Alter, T.; Lücker, E. Development of a PCR approach for differentiation of *Alaria* spp. mesocercariae. *Parasitol. Res.* **2011**, *108*, 1327–1332. [CrossRef]
23. Bilska-Zając, E.; Marucci, G.; Piróg-Komorowska, A.; Cichocka, M.; Różycki, M.; Karamon, J.; Sroka, J.; Bełcik, A.; Mizak, I.; Cencek, T. Occurrence of *Alaria alata* in wild boars (*Sus scrofa*) in Poland and detection of genetic variability between isolates. *Parasitol. Res.* **2020**, *20*, 83–91. [CrossRef]
24. Mayer-Scholl, A.; Murugaiyan, J.; Neumann, J.; Bahn, P.; Reckinger, S.; Nöckler, K. Rapid Identification of the Foodborne Pathogen *Trichinella* spp. by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *PLoS ONE* **2016**, *11*, e0152062. [CrossRef] [PubMed]
25. Wieser, A.; Schneider, L.; Jung, J.; Schubert, S. MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). *Appl. Microbiol. Biotechnol.* **2012**, *93*, 965–974. [CrossRef]
26. Shannon, S.; Kronemann, D.; Patel, R.; Schuetz, A.N. Routine use of MALDI-TOF MS for anaerobic bacterial identification in clinical microbiology. *Anaerobe* **2018**, *54*, 191–196. [CrossRef]
27. Croxatto, A.; Prod'hom, G.; Greub, G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* **2012**, *36*, 380–407. [CrossRef]
28. Bredtmann, C.M.; Krücken, J.; Murugaiyan, J.; Kuzmina, T.; von Samson-Himmelstjerna, G. Nematode species identification—current status, challenges and future perspectives for Cyathostomins. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 283. [CrossRef] [PubMed]
29. Marzano, V.; Pane, S.; Foglietta, G.; Levi Mortera, S.; Vernocchi, P.; Onetti Muda, A.; Putignani, L. Mass Spectrometry Based-Proteomic Analysis of *Anisakis* spp.: A Preliminary Study towards a New Diagnostic Tool. *Genes* **2020**, *11*, 693. [CrossRef]
30. Sy, I.; Margardt, L.; Ngbede, E.O.; Adah, M.I.; Yusuf, S.T.; Keiser, J.; Utzinger, J.; Poppert, S.; Becker, S.L. Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. *Microorganisms* **2021**, *9*, 82. [CrossRef]
31. Huguenin, A.; Depaquit, J.; Villena, I.; Ferté, H. MALDI-TOF mass spectrometry: A new tool for rapid identification of cercariae (Trematoda, Digenea). *Parasite* **2019**, *26*, 11. [CrossRef]
32. Karadjian, G.; Bilska-Zając, E.; Bahn, P.; Py, J.-S.; Johne, A.; Gassilloud, B.; Różycki, M.; Cencek, T.; Mayer-Scholl, A.; Vallée, I. Species identification of *Trichinella* originated from various host and different geographical location by MALDI-TOF. *Exp. Parasitol.* **2020**, *213*, 107890. [CrossRef] [PubMed]
33. Ozoliņa, Z.; Bagrade, G.; Deksne, G. First confirmed case of *Alaria alata* mesocercaria in Eurasian lynx (Lynx lynx) hunted in Latvia. *Parasitol. Res.* **2020**, *119*, 759–762. [CrossRef] [PubMed]
34. Ozoliņa, Z.; Deksne, G.; Pupins, M.; Gravele, E.; Gavarane, I.; Kirjušina, M. *Alaria alata* mesocercariae prevalence and predilection sites in amphibians in Latvia. *Parasitol. Res.* **2020**, *120*, 145–152. [CrossRef] [PubMed]
35. Guillen, J. FELASA guidelines and recommendations. *J. Am. Assoc. Lab. Anim. Sci.* **2012**, *51*, 311–321. [PubMed]

36. Justine, J.-L.; Briand, M.J.; Bray, R.A. A quick and simple method, usable in the field, for collecting parasites in suitable condition for both morphological and molecular studies. *Parasitol. Res.* **2012**, *111*, 341–351. [CrossRef] [PubMed]
37. Khalil, M.I.; El-Shahawy, I.S.; Abdelkader, H.S. Studies on some fish parasites of public health importance in the southern area of Saudi Arabia. *Rev. Bras. Parasitol. Veterinária* **2014**, *23*, 435–442. [CrossRef] [PubMed]
38. Olson, P.D.; Cribb, T.; Tkach, V.; Bray, R.; Littlewood, D. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int. J. Parasitol.* **2003**, *33*, 733–755. [CrossRef]
39. Portier, J.; Jouet, D.; Vallee, I.; Ferté, H. Detection of *Planorbis planorbis* and *Anisus vortex* as first intermediate hosts of *Alaria alata* (Goeze, 1792) in natural conditions in France: Molecular evidence. *Vet. Parasitol.* **2012**, *190*, 151–158. [CrossRef]
40. Marucci, G.; Interisano, M.M.; La Rosa, G.; Pozio, E. Molecular identification of nematode larvae different from those of the *Trichinella* genus detected by muscle digestion. *Vet. Parasitol.* **2013**, *194*, 117–120. [CrossRef]

Chapter 7: Discussion

7.1 Background

Consumption of raw or undercooked wild boar meat contaminated with foodborne parasites can pose a risk for human infection (Ruiz-Fons 2017). One of these parasites is the nematode *Trichinella* spp. which can cause severe disease in humans (Kocięcka 2000). In order to prevent human infections with this foodborne pathogen, official *Trichinella* inspection is mandatory for all susceptible animals intended for human consumption, in dependence of the epidemiological situation in the respective member states of the European Union (European Regulation (EU) No. 2015/1375 (European Commission 2015) combined with European Regulation (EU) No. 2020/1478 (European Commission 2020) and European Regulation (EU) No. 2021/519 (European Commission 2021)). During the *Trichinella* examinations of wild boar meat, a great diversity of larval helminths including nematodes other than *Trichinella* spp. as well as mesocercariae of the trematode *A. alata* were commonly detected in the past few years in Germany and other European countries (Möhl et al. 2009, Marucci et al. 2013, Portier et al. 2014, Gavrilović et al. 2019, Karadjian et al. 2020b). As underlined in the literature review of this thesis (chapter 2), some nematode species found during official laboratory-based meat inspection are zoonotic (e.g. *To. canis/cati, Ascaris suum, U. stenocephala*) or at least have zoonotic potential (e.g. *Metastrongylus* spp.). Also, the trematode *A. alata* which is found more and more frequently during mandatory *Trichinella* testing is classified as a zoonotic pathogen (Gottstein 2013, Federal Ministry of Labour and Social Affairs 2020).

For this reason, a reliable identification of these larval helminths is of great importance in order to understand the consumer risk associated with the consumption of wild boar meat. However, at the beginning of this thesis, standardized detection methods for the identification of incidental findings that occurred during official *Trichinella* examinations were not available. Further, recent data on the prevalence of AM in wild boars were scarce.

Therefore, the first aim of this cumulative thesis was to establish a morphology- and molecular-based assay for a reliable identification of larval nematodes potentially found during mandatory *Trichinella* inspection of wild boar meat.

With the focus on AM, the second aim of this work was to perform a prevalence study on the occurrence of AM in wild boars in the German federal state of Brandenburg to gain current data on this parasite in wild boars in an area which previously showed comparatively higher AM prevalences than other regions in Germany (Riehn et al. 2012, BVL 2016).

Finally, the third and main aim of this work was to develop a standardized technique for a rapid and reliable identification of AM in wild boar meat by MALDI-TOF mass spectrometry, which is further applicable in routine laboratories using MALDI-TOF MS as a diagnostic tool. In the longer term, this assay will contribute to improve the data situation regarding the occurrence of AM in wild boar meat.

7.2 Development of a method for identification of larval nematodes other than *Trichinella* found during mandatory *Trichinella* testing

To be able to identify a broad spectrum of larval nematodes other than *Trichinella*, an approach including both morphological examination and molecular methods targeting different genes (18S, *cox I*, ITS2, 28S) were selected.

In the first step, the found nematode larvae were morphologically examined to classify them into a taxonomic family (e.g. *Ascaridae*, *Toxocaridae*) or even genus (e.g. *Metastrongylus*) if morphological features were available. If larvae could not be categorized into a taxonomic group by morphological inspection due to a lack of morphological criteria or a degraded structure after artificial digestion, these nematodes were examined by 18S PCR followed by sequence analysis (Karadjian et al. 2020b).

The 18S PCR is based on the amplification of a 650 base pair (bp) fragment of the 18S ribosomal RNA (rRNA) gene (Karadjian et al. 2020b). As a great advantage, sequence analysis of this 18S gene sequence enables orientation of unknown nematodes towards a taxonomic group such as the genera *Ascaris*, *Toxocara*, *Metastrongylus*, *Angiostrongylus*, the family *Metastrongylidae* and *Ancylostomatidae* as well as the order *Strongylida* and *Rhabditida.* Moreover, sequence analysis of this 18S gene fragment even allows identification of trematode isolates such as AM and *Opisthioglyphe ranae* larvae, demonstrating an identity of up to 99% (Kästner et al. 2021). However, in the case of larval nematodes, the 18S gene amplification only allows for a rough classification towards a taxonomic group. Therefore, a more specific PCR targeting appropriate genes such as ITS2, *cox I* and 28S needed to be performed in a next step.

The ITS2 PCR is based on the amplification of a 500 bp gene sequence consisting of a part of the 5.8S rRNA gene and the complete internal transcribed spacer 2 (ITS2) rRNA gene (Karadjian et al. 2020b). As a major advantage of this molecular method, this PCR allows for the genus and species identification of nematodes that belong to the families *Ascaridae* and *Toxocaridae*. However, this PCR requires a prior orientation towards these taxonomic families which makes a previous careful morphological examination or the 18S PCR necessary.

The *cox I* PCR is based on the amplification of an 1100 bp partial gene fragment of the mitochondrial cytochrome oxydase I (*cox I*) gene (Karadjian et al. 2020b). This PCR enables the identification of nematodes belonging to the family *Metastrongylidae*. However, as already described for the ITS2 PCR, the *cox I* PCR also involves a prior classification towards this taxonomic family, indicating either a previous microscopic examination by morphological criteria or, if this is not possible, the implementation of the 18S PCR.

The 28S PCR is based on the amplification of a 2500 bp 28S rRNA gene fragment including the complete internal transcribed spacer 1 (ITS1), the complete 5.8S rRNA gene and the complete ITS2 gene (Karadjian et al. 2020b). This 28S PCR allows for the specific identification

of nematodes that belong to the order *Strongylida* and *Rhabditida.* However, also for this 28S PCR, a prior taxonomic classification towards these orders is necessary due to its specificity.
The first step of the described identification method is based on morphological examination of the individual isolate, indicating the need of a special knowledge regarding helminthic morphology. However, this step can easily be replaced by an 18S PCR, which makes it practicable for every technician and does not require a professional expertise in parasite's morphology. Nevertheless, for routine use of this method, it is highly recommended to deepen the knowledge of morphological characteristics at least of the most frequently found parasitic larvae, since it takes some time to obtain the results of the 18S sequence analysis before another more specific PCR targeting genes such as ITS2, *cox I* and 28S followed by sequence analysis can be performed.
Further, there is no guarantee that all isolates can be identified by this assay due to possible structural damage and DNA degradation of some larvae which can be caused by artificial digestion (Marucci et al. 2013, Karadjian et al. 2020b). However, if the amplification of the ITS2, *cox I* or 28S gene is successful, this standardized approach allows a reliable identification of nematode larvae. In addition, the 18S PCR even enables the identification of trematodes as presented in chapter 6 of this thesis. Therefore, the greatest advantage of this newly developed method is the capability to identify not only nematodes (chapter 4) as mentioned above, but also trematodes (chapter 6) such as AM, indicating a feasible identification of a broad spectrum of zoonotic and non- zoonotic helminths found during artificial digestion. This in turn will have a positive impact on the currently scarce data regarding the occurrence and frequency of a broad spectrum of helminthic parasites in wild boars, which further allows an improved assessment of potential health risks for consumers of wild boar meat.

7.3 Development of a MALDI-TOF-based method for identification of *Alaria alata* mesocercariae

The main aim of this thesis was to develop a standardized, MALDI-TOF-based technique for AM detection in wild boar meat and therefore to expand the spectrum of available diagnostic techniques. This method allows a rapid identification of AM isolated from infested carcasses in diagnostic laboratories and further enables the generation of recent data regarding both prevalence and epidemiology of AM in wild boars in regions respectively countries of interest. These findings might allow a comprehensive overview regarding the occurrence and spread of AM in different host animals and various regions and might further be useful to better estimate the risk for consumers to consume AM infested wild boar meat from different hunting areas.

In the first step of method development, a MALDI-TOF protein extraction protocol was designed which was based on the use of ten AM. This protocol enabled the generation of high-quality single spectra which were used for creation of main spectra profiles (MSPs). Finally, a total of 61 MSPs representing one host individual each were stored in a newly created AM-specific reference spectra database, which will continuously be extended and made available to other diagnostic laboratories. The main spectra included in this database allowed the comparison with those protein spectra from AM samples found during routine *Trichinella* inspection, leading to a rapid and reliable identification of these routine samples.

In a next step, this newly developed protein extraction protocol was optimized for the use of one single *A. alata* mesocercaria in order to facilitate the application in routine diagnostic laboratories equipped with MALDI-TOF mass spectrometry.

However, the implementation of a MALDI-TOF technique in routine diagnostics requires the expensive purchase of a MALDI-TOF machine including all related equipment and software. Further, an intensive training regarding the handling of this machine as well as potential sources of error is highly recommended for all users.

Nevertheless, once MALDI-TOF equipment is available in diagnostic laboratories, this newly developed method presented in this thesis (chapter 6) allows for a fast, easy and cost-efficient identification of a very broad panel of pathogens including AM. Concretely, it takes only one hour from detection of AM by stereomicroscopy to the result generated by MALDI-TOF MS (identification/confirmation). By contrast, molecular methods take minimum eight hours until the final result is available. For this reason, the short duration of this MALDI-TOF technique is a great advantage compared to the *Alaria* spp.-specific PCR (Riehn et al. 2011) and the 18S PCR described in chapter 4 of this thesis. Further, this MALDI-TOF assay was optimized for the use of only one single *A. alata* mesocercaria and therefore requires as little sample material as molecular methods do.

Moreover, this MALDI-TOF technique enables inter-species differentiation as it allows not only the identification of AM, but also the differentiation from other larval trematodes such as *Opisthioglyphe ranae*, demonstrating the high sensitivity of this method. Further, this method is suitable for intra-species differentiation which is based on cluster analysis. Here, one option is the interpretation of a MSP dendrogram which is automatically created by MALDI Biotyper 3 software and can therefore rapidly and easily be accessed by users. Another option would be to compare all relevant main spectra profiles using MALDI Biotyper 3 software and convert the generated log score values into a cross table. Based on this cross table, a heat map of all relevant MSPs can be created, displaying the cutoff values with different colors. Even though this sort of cluster analysis is clearly more time- and labor-intensive for operators than the MSP dendrogram, it is nevertheless a very illustrating way to demonstrate similarities and dissimilarities between different AM isolates.

However, the greatest advantage of this approach is the capability of a very fast and easy identification of routine AM samples without the need of any professional expertise in parasitology, indicating the application in all diagnostic laboratories equipped with MALDI-TOF mass spectrometry. Since many diagnostic laboratories have already implemented MALDI-TOF MS in the past years, this method presented in this thesis (chapter 6) might widely be applied in routine diagnostics.

However, in the field of foodborne parasitology, only two studies showed the use of MALDI-TOF MS in the framework of mandatory *Trichinella* testing (Mayer-Scholl et al. 2016, Karadjian et al. 2020a). For this reason, the MALDI-TOF technique presented in this thesis can be seen as a further step in the establishment of MALDI-TOF MS in diagnostic parasitology and might be practiced for routine examination of wild boar carcasses in future. Moreover, this assay enables a reliable identification not only of AM, but also of *Trichinella* species detected during official laboratory-based meat inspection. Currently, the caveat remains that different protein extraction protocols are needed for the preparation of e.g. AM and *Trichinella* spp. for MALDI-TOF analysis. First investigations have shown that *T. spiralis* and *T. pseudospiralis* can be reliably determined by the use of the newly developed protein extraction protocol which was initially designed for AM detection (unpublished data). Future steps will be to adapt this MALDI-TOF assay for identification of other *Trichinella* species occurring in Europe and several other helminths (e.g. *To. cati/canis*, *Metastrongylus* spp., *Ascaris suum*, *U. stenocephala*) incidentally found during mandatory *Trichinella* testing. Concretely, the aim is to create a general protein extraction protocol for identification of a variety of parasites by MALDI-TOF MS and to set up a universal database based on reference spectra from all *Trichinella* species that occur in Europe and further helminths incidentally found during routine *Trichinella* testing. This universal method will enable all diagnostic laboratories applying MALDI-TOF MS a rapid identification of a multitude of larval helminths observed during official *Trichinella* inspection. Subsequently, new findings provided by the laboratories will extend and improve the data situation regarding the occurrence and diversity of parasites in wild boar meat.

During the method developing process, MSP data from German wild boars were generated and interpreted using cluster analysis including both a heat map of all AM MSPs based on their log score values and a MSP dendrogram. In both figures presented in chapter 6 of this thesis, two clusters with different geographical origin of AM respectively of their host animals were observed. Concretely, the heat map showed a large German cluster containing only wild boar spectra from Germany and Poland as well as a small Latvian cluster including all Latvian MSPs from four different host species (wild boar, common frog, European water frog, Eurasian lynx). In the MSP dendrogram, the two clusters were presented more clearly. Here, the small cluster was formed by only Latvian spectra from wild boars, common frogs and the Eurasian lynx. The big cluster consisted of two subclusters. While the first subcluster contained both Latvian and

German main spectra from wild boars and water frogs, the second subcluster included only wild boar spectra from Germany and Poland. Consequently, this cluster formation indicated a potential variation within the *A. alata* species, demonstrating a tentative association with the geographical origin of the host, but not the host species. However, at this point, the possibility to identify intra-species variability is purely speculative due to the small size of samples investigated in this study. For this reason, it is highly desirable to constantly extend the AM-specific MSP database which is the basis for further cluster analyses and interpretation of epidemiological data of AM. For this purpose, the MALDI-TOF protocol and the AM-specific database described in chapter 6 of this thesis will be made available to diagnostic laboratories, which will further contribute to generate current prevalence and epidemiological data on AM and continuously extend the AM-specific MSP library.

7.4 Interpretation of the prevalence data of *Alaria alata* mesocercariae in wild boars from the German federal state of Brandenburg

In addition, the collection of current prevalence data on AM in wild boars was another aim of this thesis. Therefore, a prevalence study on AM in wild boars in the German federal state of Brandenburg (chapter 5) was conducted to generate long-term prevalence data on AM and better assess the risk for consumers to consume AM infested wild boar meat. The federal state of Brandenburg was selected because of the comparably high AM prevalence observed in this state during the national zoonosis monitoring in 2015 (BVL 2016).

In the survey, a total AM prevalence of 28.3% (100/354) was observed. The AM prevalences in the examined counties ranged from 11.5% (3/26) to 64.1% (25/39). In one county, no AM (0/16) were found. The latter might be due to the small size of samples from this county, indicating that the AM occurrence observed here needs to be further verified before interpreted. To analyze the wide variations between the AM prevalences in the remaining counties, these prevalence data were compared with data on the existing water areas in the examined counties of Brandenburg, which were derived from another ongoing project. This seemed relevant due to the life cycle of *A. alata* which includes water-associated intermediate hosts (water snail, amphibian). These first investigations suggest that the regional AM prevalences in wild boars correlate with the water surface in the respective area, especially in the county Uckermark where AM were highly prevalent (personal communication Dr. Rafael Mateus-Vargas, BfR). These observations are in line with findings during a prevalence study on AM in wild boars in the Free State of Saxony (Dolle 2016). Here, Dolle (2016) found a strong correlation between the water surface and the AM prevalence in the examined districts of Saxony. In future studies, the water surface of individual territories within the single counties will be compared with the regional AM prevalences for a more detailed analysis of the investigated area. Also, this analysis could be transferred to further territories not included in our study based on their

surface water areas. This in turn could allow a more precise assessment of the risk for consumers to consume meat or meat products from AM infested wild boars shot in an individual territory.

In this study, relatively high AM prevalences were determined compared to most other European studies (Paulsen et al. 2012, Paulsen et al. 2013, Portier et al. 2014, Gazzonis et al. 2018, Gavrilović et al. 2019, Bilska-Zając et al. 2020). This fact might be due to the richness of waterbodies and wetlands in Brandenburg (Knittel 2020), which offers an ideal foundation for development and spread of *A. alata* due to its water-associated life cycle (Odening 1961). Supporting this hypothesis, several authors assumed that regionally differing AM prevalences might be due to a variety of landscapes such as the presence or absence of watery areas and wetlands (Paulsen et al. 2013, Portier et al. 2014, Bilska-Zając et al. 2020, Strokowska et al. 2020).

Furthermore, the AM prevalence in amphibians such as water frogs and brown frogs as intermediate hosts for AM might play a role in the AM infestation of wild boars. While Voelkel et al. (2019) observed a parasite burden of two to 20 AM per frog in water frogs from waters around Leipzig, Saxony, Germany, Patrelle et al. (2015) found a parasite burden up to 314 and 331 AM per frog in water frogs and brown frogs from the northeast of France, respectively. Thus, the AM prevalence in amphibians, especially the parasite burden might also be an important influence factor for the AM prevalence in wild boars.

In this study, the parasite load in wild boars ranged from zero to 908 AM per sample consisting of tongue and abdominal fat tissue. In total, the mean parasite load was 9.60 AM per animal which varied widely between the AM positive counties from 0.23 AM per animal to 62.18 AM per animal. These results were mostly mirrored by the regional AM prevalences mentioned above. For this reason, it can be assumed that the mean parasite load per animal is higher in territories with higher AM prevalences than in those were AM are less prevalent in wild boars.

Further, in our study, a statistically significant correlation between prevalence and age group of the sampled wild boars was observed (p=0.001). Here, the AM prevalence increased with age since prevalences were 19.6%, 31.5% and 45.5% in wild boars of age group 0, 1 and 2, respectively.

Therefore, based on these results, the risk for consumers to become infected with AM might be higher when consuming AM infested raw or undercooked meat either from hunting areas with higher AM prevalences or from wild boars aged 2 years or older (age group 2).

7.5 *Alaria alata* mesocercariae in German wild boars – impact on hunters, flora and fauna

Currently, based on Article 28 (6) of the Implementing Regulation (EU) 2019/627 (European Commission 2019), all wild boar carcasses tested positive for incidental findings during

mandatory *Trichinella* testing must be declared unfit for human consumption. This results in financial losses for the hunters who have to pay for the disposal of the positive carcasses (personal communication with hunters, expert discussion in October 2016, BfR). In this context, AM findings are of major importance for hunters since AM prevalences in wild boars are extremely high in particular regions of Germany. For example, the regional AM prevalence in wild boars was up to 64.1% in the federal state of Brandenburg (Uckermark) (chapter 5) and even reached nearly 90% in southern Germany in the Rhine region (personal communication with experts, 2021). Such regionally high AM prevalences in wild boars imply enormous financial losses for the hunters and might reduce their motivation to keep hunting (personal communication with hunters, expert discussion in October 2016, BfR).

Reduced hunting can result in increased wild boar densities with annual rises in population size of up to 150% (Massei and Genov 2004). However, high wild boar densities can have a substantial negative impact on animals and plants. Regarding the latter, rooting plays an important role in plant damage, with reducing herbaceous plants up to 80-95% and triggering the regional extinction of particular plant species (Bratton 1974, Howe et al. 1981, Massei and Genov 2004). Also, agricultural crops are strongly affected by high wild boar densities, especially when high-energy food is lacking, indicating a negative economic impact (Mackin 1970, Andrzejewski and Jezierski 1978, Massei and Genov 2004). Further, the detrimental effect of increasing population sizes of wild boars on the fauna is caused not only by their predation on small rodents, reptiles, amphibians, invertebrates as well as eggs from ground-nesting birds, but also by their excessive rooting which devastates the habitation of surface-tunnelling rodents (Singer et al. 1984, Massei and Genov 2004). Moreover, rising wild boar densities might lead to hampered animal disease control measures in case of an outbreak. Here, a recent example is the African swine fever that provokes serious economic losses for pig farmers and pork manufacturers (Guinat et al. 2016). Finally, growing wild boar populations might also facilitate the spread of food-borne parasites such as *Trichinella* spp. and AM, which consequently might increase the risk for consumers with regard to human health.

Therefore, it is of supreme significance to keep hunting wild boars extensively and thus to reduce their population size continuously. Further, it is highly recommended to constantly update the prevalence data of AM to better estimate the present parasite burden in German wild boar populations.

The three publications presented in this thesis represent a contribution to improve the data situation concerning helminthic parasites incidentally found during official *Trichinella* examination. Especially with regard to *A. alata*, the data situation could be significantly expanded.

In particular, the newly developed MALDI-TOF technique presented in chapter 6 of this thesis is an important tool to steadily and easily generate and expand these data and also enables

all laboratories equipped with MALDI-TOF mass spectrometry to contribute to a long-term improvement of this data situation including both prevalence and epidemiological data of AM in wild boars. In general, improved data will allow a better understanding of the significance of parasites with regard to possible consequences for consumers when consuming contaminated meat. Furthermore, these data can also provide a basis for certain decisions and recommendations for action in various areas (e.g. production, trade, hunting).

7.6 Discussion on potential use of AM infested wild boar meat for human consumption

According to Article 28 (6) of the Implementing Regulation (EU) 2019/627 (European Commission 2019), carcasses infested with parasites must be declared unfit for human consumption. This is independent of the zoonotic status of the infesting parasites.

One exception is the infestation of carcasses with the tapeworm larva *Cysticercus* spp. since *Cysticercus*-free parts of not generally infested bovine animals and *Suidae* (domestic pigs, farmed game, wild game) can be declared fit for human consumption after a cold treatment (European Commission 2019). Further, as laid down in Article 3 of the Implementing Regulation (EU) 2015/1375, meat of domestic pigs which has undergone a freezing treatment according to Annex II supervised by the competent authority may be declared fit for consumption without previous *Trichinella* testing (European Commission 2015). To date, there are no further treatment regimes for the inactivation of parasites in meat authorized by the European legislation.

Considering the detrimental effects on the environment, hunting practices and disease control as highlighted above, the potentially grave financial losses and the ethical implications of the waste of a large number of game animals, an exception to Article 28 (6) should be considered. According to ANSES (2015), a thorough cooking of meat at 74°C for 5 minutes is recommended to hunters to reduce the risk and the potential risk regarding *Trichinella* and *A. alata*, respectively, when wild boar meat is consumed. To date, this recommendation refers only to wild boar carcasses which are either used for private consumption or are directly transferred to the final consumer (sale or for free) and therefore are not mandatorily examined (ANSES 2015). However, unlike *Trichinella* spp., the zoonotic potential of AM has not been scientifically proven, although some bodies have declared it pathogenic as a precautionary measure. Nevertheless, thorough cooking might potentially be an option to declare also demonstrably AM infested meat restrictedly fit for human consumption, indicating the condition that this inactivation treatment – thorough cooking at 74°C for 5 minutes – will be applied in the consumer's household. Further, if this option should actually be considered, the information "restrictedly declared fit for human consumption" and a brief instruction of the recommended treatment should be placed on the AM infested carcasses or their products so that the decision to consume may be made by the consumers.

Chapter 8: Summary

Wild boar meat infested with foodborne parasites such as the nematode species *Trichinella* can cause human disease through consumption of raw or insufficiently cooked meat or meat products. For this reason, the Implementing Regulation (EU) No. 2015/1375 makes official *Trichinella* inspection mandatory for wild boars and other susceptible species intended for human consumption. During these examinations, larval nematodes other than *Trichinella* spp. as well as mesocercariae of the trematode *Alaria (A.) alata* appeared as incidental findings in wild boar meat in the past few years in Germany and Europe.

Amongst the broad spectrum of nematodes other than *Trichinella* spp. incidentally found during mandatory *Trichinella* testing, non-zoonotic and potentially zoonotic helminths can be identified. At the beginning of this thesis, a universal method for detection of a wide variety of larval nematodes different from the *Trichinella* genus was not available.

Therefore, the first study of this thesis targeted the development of a standardized method based on both morphological examination and molecular analysis for a reliable identification of a multitude of larval nematodes found during official *Trichinella* inspection. The first step of this approach is based on either the microscopic examination by morphological criteria or the performance of an 18S PCR in order to classify the unknown nematodes towards a taxonomic group such as the families *Ascaridae*, *Toxocaridae* and *Metastrongylida* or the order *Strongylida* and *Rhabditida.* After this first classification, a specific PCR targeting genes such as ITS2, *cox I* or 28S needs to be performed in order to identify the species of the unknown nematodes. Also, it was found that the 18S PCR allows not only for the classification of unknown nematode larvae, but also for the identification of larval trematodes such as *A. alata* mesocercariae (AM), where the focus of this thesis lies on.

As data on the prevalence of AM in wild boars from Germany are scarce, the second aim of this work was to conduct a prevalence study on AM in wild boars from the German federal state of Brandenburg to obtain long-term prevalence data and better evaluate temporal and spatial variations in this area over a longer period of time. In this survey, a total AM prevalence of 28.3% (100/354) was observed among all sampled wild boars. The AM prevalences in the examined counties ranged from 11.5% (3/26) to 64.1% (25/39). In one county, no AM (0/16) were found. Further, the parasite load ranged from zero to 908 AM per animal. In total, the mean parasite load was 9.60 AM per animal which varied widely between the AM positive counties from 0.23 AM per animal to 62.18 AM per animal. Further, a statistically significant correlation between prevalence and age group of the sampled wild boars was observed (p=0.001). Here, the AM prevalence increased with age.

To date, AM found during official *Trichinella* inspection are initially identified by morphological examination using the stereomicroscope. This preliminary diagnosis is currently confirmed by

the *Alaria* spp.-specific PCR which, however, is quite time-consuming, labor-intensive and expensive. Therefore, in the third study of this thesis, a standardized MALDI-TOF-based method for a rapid and reliable identification of AM in wild boar meat was developed. First, a MALDI-TOF protein extraction protocol based on the use of ten AM was designed. This protocol allowed for the generation of high-quality single spectra which were used for creation of main spectra profiles (MSPs). Finally, a total of 61 MSPs representing one host individual each were stored in a newly created AM-specific reference spectra database. Further, this newly established protein extraction protocol was optimized for the use of one single *A. alata* mesocercaria, which enables application in routine diagnostic laboratories.

The next step will be to adapt this MALDI-TOF assay for identification of a broad spectrum of larval helminths found during mandatory *Trichinella* testing, which further will improve the data situation regarding the occurrence and diversity of parasites in wild boar meat in the long term.

In conclusion, all three publications presented in this thesis contribute to improve the data situation concerning helminthic parasites incidentally found during official *Trichinella* examination.

Chapter 9: Zusammenfassung

Alaria alata – eine Prävalenzstudie in Brandenburg und die Entwicklung protein- und molekular-basierter Verfahren zur Identifizierung von parasitären Zufallsfunden im Rahmen der amtlichen Fleischuntersuchung

Wildschweinfleisch, welches mit Lebensmittel-assoziierten Parasiten wie der Nematoden-Spezies *Trichinella* befallen ist, kann durch den Verzehr von rohem oder unzureichend erhitztem Fleisch oder Fleischprodukten Erkrankungen beim Menschen hervorrufen.
Aus diesem Grund schreibt die Durchführungsverordnung (EU) Nr. 2015/1375 die amtliche Untersuchung auf Trichinellen für Wildschweine und andere empfängliche Tierarten vor, deren Fleisch für den menschlichen Verzehr bestimmt ist.
Bei diesen obligatorischen Untersuchungen wurden in den letzten Jahren in Deutschland und Europa neben Trichinellen auch andere larvale Nematoden sowie Mesozerkarien des Trematoden *Alaria (A.) alata*, welche auch als Duncker'scher Muskelegel (DME) bezeichnet werden, als Zufallsfunde in Wildschweinfleisch detektiert. Inmitten des breiten Spektrums dieser Zufallsfunde können sowohl nicht-zoonotische als auch potenziell zoonotische Helminthen identifiziert werden.
Zu Beginn dieser Arbeit existierte keine universelle Methode zur Identifizierung einer Vielzahl von larvalen Nematoden, die nicht dem Genus *Trichinella* angehören.
Daher zielte die erste Studie dieser Arbeit auf die Entwicklung einer standardisierten Methode ab, die sowohl auf einer morphologischen Untersuchung als auch auf einer molekularen Analyse basiert und eine zuverlässige Identifizierung einer Vielzahl von larvalen Nematoden ermöglicht, die im Rahmen der amtlichen Untersuchung auf Trichinellen als Zufallsfunde detektiert werden.
Der erste Schritt dieser Methode basiert entweder auf der mikroskopischen Untersuchung nach morphologischen Kriterien oder der Durchführung einer 18S PCR, um die unbekannten Nematoden einer taxonomischen Gruppe wie den Familien *Ascaridae*, *Toxocaridae* und *Metastrongylida* oder der Ordnung *Strongylida* und *Rhabditida* zuzuordnen. Nach dieser ersten Klassifizierung wird zur Speziesidentifizierung eine spezifische PCR zum gezielten Nachweis von Genen wie ITS2, *cox I* oder 28S durchgeführt. Die im ersten Schritt dieser universellen Methode beschriebene 18S PCR ist zudem nicht nur für die Klassifizierung unbekannter Nematoden-Larven geeignet, sondern sie ermöglicht auch die Identifizierung larvaler Trematoden wie dem DME, auf dem der Fokus dieser Dissertation liegt. Da bislang nur wenige Daten zur DME-Prävalenz bei Wildschweinen in Deutschland verfügbar sind, bestand das zweite Ziel dieser Arbeit darin, eine Prävalenzstudie zum DME bei Wildschweinen im Bundesland Brandenburg durchzuführen, um langfristige Prävalenzdaten zu erhalten sowie zeitliche und räumliche Schwankungen in dieser Region über einen längeren Zeitraum besser

bewerten zu können. In dieser Studie wurde unter allen beprobten Wildschweinen eine DME-Gesamtprävalenz von 28,3% (100/354) beobachtet. Die ermittelten Prävalenzen lagen zwischen 11,5% (3/26) und 64,1% (25/39) in den untersuchten DME-positiven Landkreisen, während in einem Landkreis keine DME (0/16) detektiert wurden. Weiterhin schwankte die Parasitenbelastung zwischen null und 908 DME pro Tier. Insgesamt lag die mittlere Parasitenbelastung bei 9,60 DME pro Tier, wobei die Werte in den DME-positiven Landkreisen stark variierten (zwischen 0,23 DME und 62,18 DME pro Tier). Außerdem wurde eine statistisch signifikante Korrelation zwischen der Prävalenz und der Altersklasse der beprobten Wildschweine festgestellt (p=0,001). Hier nahm die DME-Prävalenz mit dem Alter zu.

Bislang werden DME, die im Rahmen der amtlichen Untersuchung auf Trichinellen detektiert werden, zunächst durch die morphologische Untersuchung mittels Stereomikroskop identifiziert. Diese vorläufige Diagnose wird derzeit durch die *Alaria* spp.-spezifische PCR bestätigt, welche jedoch recht zeitaufwendig, arbeitsintensiv und mit entsprechenden Kosten verbunden ist.

Daher wurde in der dritten Studie dieser Arbeit eine standardisierte, MALDI-TOF-basierte Methode für eine schnelle und zuverlässige Identifizierung von DME in Wildschweinfleisch etabliert. Zunächst wurde ein MALDI-TOF-Proteinextraktionsprotokoll entwickelt, welches auf der Verwendung von zehn DME basiert. Dieses Protokoll ermöglichte die Erzeugung hochwertiger Einzelspektren, die für die Erstellung von Referenzspektren, sogenannten Main Spectra Profiles (MSPs), herangezogen wurden. Schließlich wurden insgesamt 61 MSPs, welche jeweils ein Wirtstierindividuum repräsentieren, in einer neu erstellten DME-spezifischen Referenzspektren-Datenbank hinterlegt, die diagnostischen Routinelaboren zur Verfügung gestellt werden kann. Darüber hinaus wurde dieses neu entwickelte Proteinextraktionsprotokoll für den Einsatz eines einzigen DME optimiert, was die Anwendung in diagnostischen Routinelaboratorien ermöglicht.

Im nächsten Schritt soll diese MALDI-TOF-basierte Methode dahingehend adaptiert werden, dass dadurch die Identifizierung eines breiten Spektrums an larvalen Helminthen, welche bei der amtlichen Untersuchung auf Trichinellen detektiert werden, ermöglicht wird. Dies wiederum wird langfristig die Datenlage hinsichtlich des Vorkommens und der Vielfalt von Parasiten in Wildschweinfleisch verbessern.

Zusammenfassend betrachtet leisten alle drei Publikationen, die in dieser Doktorarbeit vorgestellt wurden, einen wertvollen Beitrag zur Verbesserung der Datenlage in Bezug auf Helminthen, welche im Rahmen der amtlichen Untersuchung auf Trichinellen als Zufallsfunde detektiert werden.

References

Al-Sabi, M. N. S., M. Chriél, T. H. Jensen and H. L. Enemark (2013). "Endoparasites of the raccoon dog (*Nyctereutes procyonoides*) and the red fox (*Vulpes vulpes*) in Denmark 2009–2012 – A comparative study." International Journal for Parasitology: Parasites and Wildlife 2: 144-151.

Andrzejewski, R. and W. Jezierski (1978). "Management of a wild boar population and its effects on commercial land." Acta theriologica 23(19): 309-339.

ANSES (2015). "Opinion of the French Agency for Food, Environmental and Occupational Health & Safety on the Presence of Mesocercarial Parasites of the Trematode *Alaria alata* in Wild Boar Meat (Request no 2015-SA-0052, 33 pp.)."

Astrup, A. (1945). "*Uncinaria Stenocephala* as a Cause of Skin Disease in Man." Acta Dermato-Venereologica 25(5): 389-392.

Beattie, P. and C. Fleming (2002). "Cutaneous larva migrans in the west coast of Scotland." Clinical and Experimental Dermatology 27(3): 248-249.

Berger, E. and P. Paulsen (2014). "Findings of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*, Linnaeus, 1758) in west Hungary (Transdanubia regions)." Wiener Tierärztliche Monatsschrift 101(5/6): 120-123.

BfR (2017). "Wildschweinfleisch kann den Duncker´schen Muskelegel enthalten." Aktualisierte Stellungnahme Nr. 011/2017 des BfR vom 27. Juni 2017*.

Bilska-Zając, E., G. Marucci, A. Piróg-Komorowska, M. Cichocka, M. Różycki, J. Karamon, J. Sroka, A. Bełcik, I. Mizak and T. Cencek (2020). "Occurrence of *Alaria alata* in wild boars (*Sus scrofa*) in Poland and detection of genetic variability between isolates." Parasitology Research (2021) 120:83-91.

Blackwell, V. and F. Vega-Lopez (2001). "Cutaneous larva migrans: clinical features and management of 44 cases presenting in the returning traveller." British Journal of Dermatology 145(3): 434-437.

Bolt, G., J. Monrad, J. Koch and A. Jensen (1994). "Canine angiostrongylosis: a review." The Veterinary Record 135(19): 447-452.

Borgsteede, F. (1984). "Helminth parasites of wild foxes (*Vulpes vulpes* L.) in The Netherlands." Zeitschrift für Parasitenkunde 70(3): 281-285.

Bowman, D. D., S. P. Montgomery, A. M. Zajac, M. L. Eberhard and K. R. Kazacos (2010). "Hookworms of dogs and cats as agents of cutaneous larva migrans." Trends in Parasitology 26(4): 162-167.

Bratton, S. P. (1974). "The effect of the European wild boar (*Sus scrofa*) on the high-elevation vernal flora in Great Smoky Mountains National Park." Bulletin of the Torrey Botanical Club: 198-206.

Bredtmann, C. M., J. Krücken, J. Murugaiyan, T. Kuzmina and G. von Samson-Himmelstjerna (2017). "Nematode species identification – current status, challenges and future perspectives for Cyathostomins." Frontiers in Cellular and Infection Microbiology 7: 283.

Bružinskaitė-Schmidhalter, R., M. Šarkūnas, A. Malakauskas, A. Mathis, P. R. Torgerson and P. Deplazes (2012). "Helminths of red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) in Lithuania." Parasitology 139(1): 120-127.

BVL (2016). "Zoonosen-Monitoring 2015. Gemeinsamer Bericht des Bundes und der Länder. Berichte zur Lebensmittelsicherheit, BVL-Report 11.2: 30-31."

Calderaro, A., M.-C. Arcangeletti, I. Rodighiero, M. Buttrini, C. Gorrini, F. Motta, D. Germini, M.-C. Medici, C. Chezzi and F. De Conto (2014). "Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification." Scientific Reports 4: 6803.

Calderaro, A., M. C. Arcangeletti, I. Rodighiero, M. Buttrini, S. Montecchini, R. Vasile Simone, M. C. Medici, C. Chezzi and F. De Conto (2016). "Identification of different respiratory viruses, after a cell culture step, by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS)." Scientific Reports 6(1): 36082.

Calvopina, M., H. Caballero, T. Morita and M. Korenaga (2016). "Human pulmonary infection by the zoonotic *Metastrongylus salmi* Nematode. The first reported case in the Americas." The American Journal of Tropical Medicine and Hygiene 95(4): 871-873.

Castagna, F., V. Musella, L. Esposito, A. Poerio, L. Rinaldi, A. Bosco, G. Cringoli and D. Britti (2019). "Helminths of wild boar (*Sus scrofa*) in the Calabrian region of Southern Italy." Journal of Wildlife Diseases 55(2): 416-420.

Castro, O., J. Venzal and M. Felix (2009). "Two new records of helminth parasites of domestic cat from Uruguay: *Alaria alata* (Goeze, 1782) (*Digenea*, *Diplostomidae*) and *Lagochilascaris major* (Leiper, 1910) *(Nematoda*, *Ascarididae*)." Veterinary Parasitology 160(3-4): 344-347.

Caumes, E. and M. Danis (2004). "From creeping eruption to hookworm-related cutaneous larva migrans." The Lancet. Infectious Diseases 4(11): 659-660.

Crewe, W. and D. H. Smith (1971). "Human infection with pig *Ascaris* (*A. suum*)." Annals of Tropical Medicine and Parasitology 65(1).

Croxatto, A., G. Prod'hom and G. Greub (2012). "Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology." FEMS Microbiol Rev 36(2): 380-407.

Davidson, W. R., M. J. Appel, G. L. Doster, O. E. Baker and J. F. Brown (1992). "Diseases and parasites of red foxes, gray foxes, and coyotes from commercial sources selling to fox-chasing enclosures." Journal of Wildlife Diseases 28(4): 581-589.

Davies, H. D., P. Sakuls and J. S. Keystone (1993). "Creeping eruption: A review of clinical presentation and management of 60 cases presenting to a tropical disease unit." Archives of Dermatology 129(5): 588-591.

De-La-Muela, N., S. Hernández-de-Luján and I. Ferre (2001). "Helminths of wild boar in Spain." Journal of Wildlife Diseases 37(4): 840-843.

Demirev, P. A., Y.-P. Ho, V. Ryzhov and C. Fenselau (1999). "Microorganism identification by mass spectrometry and protein database searches." Analytical Chemistry 71(14): 2732-2738.

Deplazes, P., J. Eckert, v. Samson-Himmelstjerna and H. Zahner (2012). "Lehrbuch der Parasitologie für die Tiermedizin. 3. überarbeitete Auflage." Enke Verlag, Stuttgart.

Diba, V., C. Whitty and T. Green (2004). "Cutaneous larva migrans acquired in Britain." Clinical and Experimental Dermatology 29(5): 555-556.

Dolle, S. (2016). "Prävalenz und geographische Verteilung des Duncker´schen Muskelegels (*Alaria alata*-Mesozerkarie) in Wildschweinen (*Sus scrofa*) im Freistaat Sachsen."

Duncker, H. C. J. (1896). "Die Muskeldistomeen." Berliner Tierärztliche Wochenschrift Nr. 24: 279-282.

Duprez, T., G. Bigaignon, E. Delgrange, P. Desfontaines, M. Hermans, T. Vervoort, C. Sindic and M. Buysschaert (1996). "MRI of cervical cord lesions and their resolution in *Toxocara canis* myelopathy." Neuroradiology 38(8): 792-795.

Duscher, G. (2011). "Der Dunckersche Muskelegel – *Alaria alata* beim Rotfuchs in Österreich in Relation zum Vorkommen von Wildschweinen." Wiener Tierärztliche Monatsschrift 98: 251-254.

EFSA (2018). "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017." EFSA Journal 16(12): e05500.

Eira, C., J. Vingada, J. Torres and J. Miquel (2006). "The helminth community of the red fox, *Vulpes vulpes*, in Dunas de Mira (Portugal) and its effect on host condition." Wildlife Biology in Practice, ISSN: 1646-2742.

European Commission (2005).”Commission Regulation (EC) No 2075/2005 of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat.” E. Commission. Official Journal of the European Union.

European Commission (2015).”Commission Implementing Regulation (EU) 2015/1375 of 10 August 2015 laying down specific rules on official controls for *Trichinella* in meat.” E. Commission. Official Journal of the European Union.

European Commission (2019).”Commission Implementing Regulation (EU) 2019/627 of 15 March 2019 laying down uniform practical arrangements for the performance of official controls on products of animal origin intended for human consumption in accordance with Regulation (EU) 2017/625 of the European Parliament and of the Council and amending Commission Regulation (EC) No 2074/2005 as regards official controls.” E. Commission. Official Journal of the European Union.

European Commission (2020).”Commission Implementing Regulation (EU) 2020/1478 of 14 October 2020 amending Implementing Regulation (EU) 2015/1375 as regards sampling, the reference method for detection and import conditions related to *Trichinella* control.” E. Commission. Official Journal of the European Union.

European Commission (2021).”Commission Implementing Regulation (EU) 2021/519 of 24 March 2021 amending Implementing Regulation (EU) 2015/1375 as regards testing for *Trichinella* in solipeds and the derogation of the United Kingdom from testing for *Trichinella* in domestic swine.” E. Commission. Official Journal of the European Union.

Federal Ministry of Labour and Social Affairs (2020). "Technische Regeln für Biologische Arbeitsstoffe (TRBA) 464 "Einstufung von Parasiten in Risikogruppen", Ausgabe Juli 2013, 1. Änderung vom 10.11.2020, GMBI Nr. 45."

Fenselau, C. and P. A. Demirev (2001). "Characterization of intact microorganisms by MALDI mass spectrometry." Mass Spectrometry Reviews 20(4): 157-171.

Fernandes, B., J. Cooper, J. Cullen, R. Freeman, A. Ritchie, A. Scott and P. Stuart (1976). "Systemic infection with *Alaria americana* (*Trematoda*)." Canadian Medical Association Journal 115(11): 1111.

Forbes, L. B., S. Parker and W. B. Scandrett (2003). "Comparison of a modified digestion assay with trichinoscopy for the detection of *Trichinella* larvae in pork." Journal of Food Protection 66(6): 1043-1046.

Freeman, R. S., P. F. Stuart, J. B. Cullen, A. C. Ritchie, A. Mildon, B. J. Fernandes and R. Bonin (1976). "Fatal human infection with mesocercariae of the trematode *Alaria americana*." American Journal of Tropical Medicine and Hygiene 25(6): 803-807.

Galanti, B., F. M. Fusco and S. Nardiello (2002). "Outbreak of cutaneous larva migrans in Naples, southern Italy." Transactions of the Royal Society of Tropical Medicine and Hygiene 96(5): 491-492.

Galvin, T. J. (1968). "Development of human and pig *Ascaris* in the pig and rabbit." The Journal of Parasitology: 1085-1091.

Gamble, H. R., A. S. Bessonov, K. Cuperlovic, A. A. Gajadhar, F. van Knapen, K. Noeckler, H. Schenone and X. Zhu (2000). "International Commission on Trichinellosis: recommendations on methods for the control of *Trichinella* in domestic and wild animals intended for human consumption." Vet Parasitol 93(3-4): 393-408.

García-González, Á. M., J. E. Pérez-Martín, J. A. Gamito-Santos, R. Calero-Bernal, M. A. Alonso and E. M. F. Carrión (2013). "Epidemiologic study of lung parasites (*Metastrongylus* spp.) in wild boar (*Sus scrofa*) in southwestern Spain." Journal of Wildlife Diseases 49(1): 157-162.

Gavrilović, P., I. Pavlović and I. Todorović (2019). "*Alaria alata* mesocercariae in domestic pigs and wild boars in South Banat, northern Serbia." Comp Immunol Microbiol Infect Dis 63: 142-144.

Gazzonis, A. L., L. Villa, K. Riehn, A. Hamedy, S. Minazzi, E. Olivieri, S. A. Zanzani and M. T. Manfredi (2018). "Occurrence of selected zoonotic food-borne parasites and first molecular identification of *Alaria alata* in wild boars (*Sus scrofa*) in Italy." Parasitol Res 117(7): 2207-2215.

Giebel, R., C. Worden, S. M. Rust, G. T. Kleinheinz, M. Robbins and T. R. Sandrin (2010). Chapter 6 - Microbial Fingerprinting using Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS): Applications and Challenges. Advances in Applied Microbiology, Academic Press. 71: 149-184.

Gillespie, S., W. Dinning, A. Voller and N. Crowcroft (1993). "The spectrum of ocular toxocariasis." Eye 7(3): 415-418.

Glickman, L. T., J.-F. Magnaval, L. M. Domanski, F. S. Shofer, S. S. Lauria, B. Gottstein and B. Brochier (1987). "Visceral larva migrans in French adults: A new disease syndrome?" American Journal of Epidemiology 125(6): 1019-1034.

Glickman, L. T. and P. M. Schantz (1981). "Epidemiology and pathogenesis of zoonotic toxocariasis." Epidemiologic Reviews 3: 230-250.

Goeze, J. A. E. (1782).“Versuch einer Naturgeschichte der Eingeweidewürmer thierischer Körper.“ Leipzig: Bei Siegfried Lebrecht Crusius, 1782.
Retrieved from https://www.digi-hub.de/viewer/image/BV043819491/5/#topDocAnchor.

González-Fuentes, H., A. Hamedy, M. Koethe, E. von Borell, E. Luecker and K. Riehn (2015). "Effect of temperature on the survival of *Alaria alata* mesocercariae." Parasitology Research 114(3): 1179-1187.

González-Fuentes, H., A. Hamedy, E. von Borell, E. Luecker and K. Riehn (2014). "Tenacity of *Alaria alata* mesocercariae in homemade German meat products." International Journal of Food Microbiology 176: 9-14.

Górski, P., A. Zalewski and M. Lakomy (2006). "Parasites of carnivorous mammals in Bialowieza Primeval Forest." Wiadomości Parazytologiczne 52(1).

Gottstein, B. (2013). Einstufung von Organismen. Modul 3: Parasiten. Stand Januar 2013. Herausgegeben vom Bundesamt für Umwelt BAFU, Bundesamt für Gesundheit BAG, Bern, Schweiz. Retrieved from http://www.bafu.admin.ch/uv-1114-d.

Guinat, C., A. Gogin, S. Blome, G. Keil, R. Pollin, D. U. Pfeiffer and L. Dixon (2016). "Transmission routes of African swine fever virus to domestic pigs: Current knowledge and future research directions." Veterinary Record 178(11): 262-267.

Herrmann, A., T. Christoph and G. Sebastian (2004). "Larva migrans "saxoniae": Larva migrans infection in Saxony." Journal of the German Society of Dermatology: JDDG 2(1): 46-48.

Hiepe, T. (1985). "Lehrbuch der Parasitologie Bd 3: Veterinärmedizinische Helminthologie." Gustav Fischer Verlag, Stuttgart.

Hill, I. R., D. Denham and C. Scholtz (1985). "*Toxocara canis* larvae in the brain of a British child." Transactions of the Royal Society of Tropical Medicine and Hygiene 79(3): 351-354.

Hobmaier, M. (1929). "Entwicklung und Invasion von *Metastrongylus Elongatus* der Lunge." Klinische Wochenschrift 8(35): 1625-1625.

Howe, T. D., F. J. Singer and B. B. Ackerman (1981). "Forage relationships of European wild boar invading northern hardwood forest." The Journal of Wildlife Management 45(3): 748-754.

Huguenin, A., J. Depaquit, I. Villena and H. Ferté (2019). "MALDI-TOF mass spectrometry: A new tool for rapid identification of cercariae (*Trematoda*, *Digenea*)." Parasite 26.

Iles, R. K., R. Zmuidinaite, J. K. Iles, G. Carnell, A. Sampson and J. L. Heeney (2020). "Development of a Clinical MALDI-TOF Mass Spectrometry Assay for SARS-CoV-2: Rational Design and Multi-Disciplinary Team Work." Diagnostics 10(10): 746.

Ilić, T., Z. Becskei, T. Petrović, V. Polaček, B. Ristić, S. Milić, P. Stepanović, K. Radisavljević and S. Dimitrijević (2016). "Endoparasitic fauna of red foxes (*Vulpes vulpes*) and golden jackals (*Canis aureus*) in Serbia." Acta Parasitologica 61(2): 389-396.

Jakšić, S., S. Uhitil and M. Vučemilo (2002). "Nachweis von Mesozerkarien des Saugwurms *Alaria alata* im Wildschweinefleisch." Zeitschrift für Jagdwissenschaft 48(3): 203-207.

Jaskoski, B. (1961). "An apparent swine *Ascaris* infection of man." Journal of the American Veterinary Medical Association 138(9): 504-505.

Jelinek, T., H. Maiwald, H. D. Nothdurft and T. Löscher (1994). "Cutaneous Larva Migrans in Travelers: Synopsis of Histories, Symptoms, and Treatment of 98 Patients." Clinical Infectious Diseases 19(6): 1062-1066.

Joshi, M. and J. Deshpande (2010). "Polymerase chain reaction: Methods, principles and application." International Journal of Biomedical Research 2(1): 81-97.

Karadjian, G., E. Bilska-Zając, P. Bahn, J.-S. Py, A. Johne, B. Gassilloud, M. Różycki, T. Cencek, A. Mayer-Scholl and I. Vallée (2020a). "Species identification of *Trichinella* originated from various host and different geographical location by MALDI-TOF." Experimental Parasitology 213: 107890.

Karadjian, G., C. Kaestner, L. Laboutière, E. Adicéam, T. Wagner, A. Johne, M. Thomas, B. Polack, A. Mayer-Scholl and I. Vallée (2020b). "A two-step morphology-PCR strategy for the identification of nematode larvae recovered from muscles after artificial digestion at meat inspection." Parasitology Research (2020) 119:4113-4122.

Kästner, C., P. Bahn, R. Schönfelder, Z. Ozoliņa, L. Alksne, M. H. Richter, G. Deksne, A. Mayer-Scholl and A. Johne (2021). "Development of a Novel Method for Identification of *Alaria alata* Mesocercariae by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry." *Microorganisms* 9(8): 1664.

Klose, C., S. Mravak, M. Geb, U. Bienzle and C. Meyer (1996). "Autochthonous cutaneous larva migrans in Germany." Tropical Medicine & International Health 1(4): 503-504.

Knittel, T. (2020). "Gewässer in Brandenburg." Retrieved from https://www.in-berlin-brandenburg.com/Brandenburg/Urlaub/Gewaesser/.

Koch, J. and J. Willesen (2009). "Canine pulmonary angiostrongylosis: An update." The Veterinary Journal 179(3): 348-359.

Kocięcka, W. (2000). "Trichinellosis: Human disease, diagnosis and treatment." Veterinary Parasitology 93(3): 365-383.

Koino, S. (1922). "Experimental Infection with *Ascaris* in Human Body, with Special Reference to the Clinical Symptoms of *Ascaris* Pneumonia. (A Preliminary Report.)." Tokyo Med. News (2299).

Komiyama, A., O. Hasegawa, S. Nakamura, S. Ohno and K. Kondo (1995). "Optic neuritis in cerebral toxocariasis." Journal of Neurology, Neurosurgery, and Psychiatry 59(2): 197-198.

Kramer, M. H., M. L. Eberhard and T. A. Blankenberg (1996). "Respiratory symptoms and subcutaneous granuloma caused by mesocercariae: A case report." Am J Trop Med Hyg 55(4): 447-448.

Kumar, J. and J. Kimm (1994). "MR in *Toxocara canis* myelopathy." American Journal of Neuroradiology 15(10): 1918-1920.

Lima, R. C., E. G. L. Hoppe, J. H. Tebaldi, B. C. Cruz, A. A. B. Gomes and A. A. Nascimento (2013). "Gastrointestinal helminths of *Cerdocyon thous* (Linnaeus, 1766-Smith, 1839) from the caatinga area of the Paraíba State, Brazil." Semina: Ciências Agrárias 34(6): 2879-2888.

Loos-Frank, B. and E. Zeyhle (1982). "The intestinal helminths of the red fox and some other carnivores in southwest Germany." Zeitschrift für Parasitenkunde 67(1): 99-113.

Lord, W. and W. Bullock (1982). "Swine *Ascaris* in humans." New England Journal of Medicine 306(18).

Lucius, R., W. Böckeler and A. S. Pfeiffer (1988). "Parasiten der Haus-, Nutz- und Wildtiere Schleswig-Holsteins: Parasiten der inneren Organe des Rotfuchses (*Vulpes vulpes*)." Zeitschrift für Jagdwissenschaft 34(4): 242-255.

Machida, M., Y. Kitamura and H. Kamiya (1975). "Occurrence of *Alaria alata* (*Diplostomidae: Digenea*) from the red fox in Hokkaido, Japan." Japanese Journal of Parasitology 24(3): 144-147.

Mackin, R. (1970). "Dynamics of damage caused by wild boar to different agricultural crops." Acta Theriologica 15(27): 447-458.

Magnaval, J.-F., L. T. Glickman, P. Dorchies and B. Morassin (2001). "Highlights of human toxocariasis." The Korean Journal of Parasitology 39(1): 1.

Marucci, G., M. Interisano, G. La Rosa and E. Pozio (2013). "Molecular identification of nematode larvae different from those of the *Trichinella* genus detected by muscle digestion." Vet Parasitol 194(2-4): 117-120.

Marzano, V., S. Pane, G. Foglietta, S. Levi Mortera, P. Vernocchi, A. Onetti Muda and L. Putignani (2020). "Mass Spectrometry Based-Proteomic Analysis of *Anisakis* spp.: A Preliminary Study towards a New Diagnostic Tool." Genes 11(6): 693.

Massei, G. and P. Genov (2004). "The environmental impact of wild boar." Galemys, 16 (nº especial): 135-145, 2004.

Mayer-Scholl, A., J. Murugaiyan, J. Neumann, P. Bahn, S. Reckinger and K. Nockler (2016). "Rapid Identification of the Foodborne Pathogen *Trichinella* spp. by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry." PLoS One 11(3): e0152062.

McDonald, H. R., K. R. Kazacos, H. Schatz and R. N. Johnson (1994). "Two cases of intraocular infection with *Alaria mesocercaria* (*Trematoda*)." Am J Ophthalmol 117(4): 447-455.

Möhl, K., K. Grosse, A. Hamedy, T. Wuste, P. Kabelitz and E. Lucker (2009). "Biology of *Alaria* spp. and human exposition risk to *Alaria* mesocercariae – a review." Parasitol Res 105(1): 1-15.

Morgan, E. and S. Shaw (2010). "*Angiostrongylus vasorum* infection in dogs: Continuing spread and developments in diagnosis and treatment." J Small Anim Pract 51(12): 616-621.

Mullis, K. B. and F. A. Faloona (1987).”[21] Specific Synthesis of DNA in Vitro via a Polymerase-Catalyzed Chain Reaction.” Methods in Enzymology, Academic Press. 155: 335-350.

Murphy, T. M., J. O'Connell, M. Berzano, C. Dold, J. D. Keegan, A. McCann, D. Murphy and N. M. Holden (2012). "The prevalence and distribution of *Alaria alata*, a potential zoonotic parasite, in foxes in Ireland." Parasitol Res 111(1): 283-290.

Nejsum, P., M. Betson, R. Bendall, S. Thamsborg and J. Stothard (2012). "Assessing the zoonotic potential of *Ascaris suum* and *Trichuris suis*: Looking to the future from an analysis of the past." Journal of Helminthology 86(2): 148-155.

Odening, K. (1961). "Der "Dunckersche Muskelegel" kann experimentell auf Affen übertragen werden." Monatshefte für Veterinärmedizin 16:395-399.

Odening, K. (1963). "Zur Diagnostik der Mesozerkarie von *Alaria alata*, eines möglichen Parasiten des Menschen in Europa, an Hand experimenteller Befunde beim Affen." Dt. Akad. Wiss., Berlin: 385-390.

Olson, P. D., T. H. Cribb, V. V. Tkach, R. A. Bray and D. T. Littlewood (2003). "Phylogeny and classification of the *Digenea* (*Platyhelminthes*: *Trematoda*)." Int J Parasitol 33(7): 733-755.

Ozoliņa, Z., G. Bagrade and G. Deksne (2018). "The host age related occurrence of *Alaria alata* in wild canids in Latvia." Parasitology Research 117(12): 3743-3751.

Ozoliņa, Z., G. Bagrade and G. Deksne (2020a). "First confirmed case of *Alaria alata* mesocercaria in Eurasian lynx (Lynx lynx) hunted in Latvia." Parasitology Research 119(2): 759-762.

Ozoliņa, Z. and G. Deksne (2017). "Effectiveness of two methods for mesocercariae of *Alaria alata* detection in wild boars (*Sus scrofa*)." Environmental and Experimental Biology 15: 25-28.

Ozoliņa, Z., G. Deksne, M. Pupins, E. Gravele, I. Gavarane and M. Kirjušina (2020c). "*Alaria alata* mesocercariae prevalence and predilection sites in amphibians in Latvia." Parasitology Research (2021) 120:145-152.

Ozoliņa, Z., M. Mateusa, L. Šuksta, L. Liepiņa and G. Deksne (2020b). "The wild boar (*Sus scrofa*, Linnaeus, 1758) as an important reservoir host for *Alaria alata* in the Baltic region and potential risk of infection in humans." Veterinary Parasitology: Regional Studies and Reports 22: 100485.

Patrelle, C., J. Portier, D. Jouet, D. Delorme and H. Ferté (2015). "Prevalence and intensity of *Alaria alata* (Goeze, 1792) in water frogs and brown frogs in natural conditions." Parasitol Res 114(12): 4405-4412.

Paulsen, P., J. Ehebruster, I. Irschik, E. Lücker, K. Riehn, R. Winkelmayer and F. J. M. Smulders (2012). "Findings of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) in eastern Austria." European Journal of Wildlife Research 58(6): 991-995.

Paulsen, P., P. Forejtek, Z. Hutarova and M. Vodnansky (2013). "*Alaria alata* mesocercariae in wild boar (*Sus scrofa*, Linnaeus, 1758) in south regions of the Czech Republic." Vet Parasitol 197(1-2): 384-387.

Pawlowski, Z. (2001). "Toxocariasis in humans: Clinical expression and treatment dilemma." Journal of Helminthology 75(4): 299.

Phillipson, R. and J. Race (1967). "Human infection with porcine *Ascaris*." British Medical Journal 3(5569): 865.

Popiołek, M., D. Knecht, J. Szczęsna-Staśkiewicz and A. Czerwińska-Rożałow (2010). "Helminths of the wild boar (*Sus scrofa* L.) in natural and breeding conditions." Bull Vet Inst Pulawy 54(2): 161-166.

Portier, J., D. Jouet, H. Ferté, O. Gibout, A. Heckmann, P. Boireau and I. Vallée (2011). "New data in France on the trematode *Alaria alata* (Goeze, 1792) obtained during *Trichinella* inspections." Parasite: Journal de la Société Française de Parasitologie 18(3): 271.

Portier, J., I. Vallée, S. A. Lacour, R. Martin-Schaller, H. Ferté and B. Durand (2014). "Increasing circulation of *Alaria alata* mesocercaria in wild boar populations of the Rhine valley, France, 2007-2011." Vet Parasitol 199(3-4): 153-159.

Pozio, E. and D. S. Zarlenga (2013). "New pieces of the *Trichinella* puzzle." International Journal for Parasitology 43(12-13): 983-997.

Ramisz, A. and A. Balicka-Ramisz (2006). "Evaluation of the meat carcasses assigned for consumption towards the presence of *Trichinella* larvae by two methods: Trichinoscopy and digestion." Wiadomosci parazytologiczne 52(3): 199-204.

Rentería-Solís, Z., M. Kołodziej-Sobocińska and K. Riehn (2018). "*Alaria* spp. mesocercariae in Eurasian badger (*Meles meles*) and wild boar (*Sus scrofa*) from the Białowieża Forest, north-eastern Poland." Parasitology Research 117(4): 1297-1299.

Rentería-Solís, Z. M., A. Hamedy, F.-U. Michler, B. A. Michler, E. Lücker, N. Stier, G. Wibbelt and K. Riehn (2013). "*Alaria alata* mesocercariae in raccoons (*Procyon lotor*) in Germany." Parasitology Research 112(10): 3595-3600.

Riehn, K., A. Hamedy, T. Alter and E. Lucker (2011). "Development of a PCR approach for differentiation of *Alaria* spp. mesocercariae." Parasitol Res 108(5): 1327-1332.

Riehn, K., A. Hamedy, K. Grosse, T. Wüste and E. Lücker (2012). "*Alaria alata* in wild boars (*Sus scrofa*, Linnaeus, 1758) in the eastern parts of Germany." Parasitol Res 111(4): 1857-1861.

Riehn, K., A. Hamedy, K. Grosse, L. Zeitler and E. Lücker (2010). "A novel detection method for *Alaria alata* mesocercariae in meat." Parasitol Res 107(1): 213-220.

Riehn, K., N. Lalkovski, A. Hamedy and E. Lücker (2014). "First detection of *Alaria alata* mesocercariae in wild boars (*Sus scrofa* Linnaeus, 1758) from Bulgaria." J Helminthol 88(2): 247-249.

Roepstorff, A., H. Mejer, P. Nejsum and S. M. Thamsborg (2011). "Helminth parasites in pigs: New challenges in pig production and current research highlights." Veterinary Parasitology 180(1): 72-81.

Roepstorff, A. and P. Nansen (1994). "Epidemiology and control of helminth infections in pigs under intensive and non-intensive production systems." Veterinary Parasitology 54(1-3): 69-85.

Ruas, J. L., G. Muller, N. Farias, T. Gallina, A. S. Lucas, F. G. Pappen, A. L. Sinkoc and J. Brum (2008). "Helminths of Pampas fox, *Pseudalopex gymnocercus* (Fischer, 1814) and of Crab-eating fox, *Cerdocyon thous* (Linnaeus, 1766) in the South of the State of Rio Grande do Sul, Brazil." Brazilian Journal of Veterinary Parasitology: Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria 17(2): 87-92.

Ruiz-Fons, F. (2017). "A Review of the Current Status of Relevant Zoonotic Pathogens in Wild Swine (*Sus scrofa*) Populations: Changes Modulating the Risk of Transmission to Humans." Transboundary and Emerging Diseases 64(1): 68-88.

Russegger, L. and E. Schmutzhard (1989). "Spinal toxocaral abscess." The Lancet 334(8659): 398.

Rüttinger, P. and H. Hadidi (1991). "MRI in cerebral toxocaral disease." Journal of Neurology, Neurosurgery, and Psychiatry 54(4): 361-362.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science 239(4839): 487-491.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim (1985). "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia." Science 230(4732): 1350-1354.

Sailer, A., W. Glawischnig, I. Irschik, E. Lücker, K. Riehn and P. Paulsen (2012). "Findings of *Alaria alata* mesocercariae in wild boar in Austria: Current knowledge, identification of risk factors and discussion of risk management options." Wiener Tierärztliche Monatsschrift 99: 346-352.

Shannon, S., D. Kronemann, R. Patel and A. N. Schuetz (2018). "Routine use of MALDI-TOF MS for anaerobic bacterial identification in clinical microbiology." Anaerobe 54: 191-196.

Shimalov, V. and V. Shimalov (2001). "Helminth fauna of the stoat (*Mustela erminea* Linnaeus, 1758) and the weasel (*M. nivalis* Linnaeus, 1758) in Belorussian Polesie." Parasitology Research 87(8): 680-681.

Shimalov, V. and V. Shimalov (2002). "Helminth fauna of the red fox (*Vulpes vulpes* Linnaeus, 1758) in southern Belarus." Parasitology Research 89(1): 77-78.

Shimalov, V., V. Shimalov and A. Shimalov (2000). "Helminth fauna of otter (*Lutra lutra* Linnaeus, 1758) in Belorussian Polesie." Parasitology Research 86(6).

Singer, F. J., W. T. Swank and E. E. Clebsch (1984). "Effects of wild pig rooting in a deciduous forest." The Journal of Wildlife Management: 464-473.

Singh, J., N. Birbian, S. Sinha and A. Goswami (2014). "A critical review on PCR, its types and applications." Int J Adv Res Biol Sci 1(7): 65-80.

Smith, H. (1978). "Parasites of red foxes in New Brunswick and Nova Scotia." Journal of Wildlife Diseases 14(3): 366-370.

Spratt, D. M. (2015). "Species of *Angiostrongylus* (*Nematoda*: *Metastrongyloidea*) in wildlife: A review." International Journal for Parasitology: Parasites and Wildlife 4(2): 178-189.

Strokowska, N., M. Nowicki, D. Klich, Z. Bełkot, J. Wiśniewski, A. Didkowska, P. Chyla and K. Anusz (2020). "The occurrence of *Alaria alata* mesocercariae in wild boars (*Sus scrofa*) in north-eastern Poland." Int J Parasitol Parasites Wildl 12: 25-28.

Sy, I., L. Margardt, E. O. Ngbede, M. I. Adah, S. T. Yusuf, J. Keiser, J. Rehner, J. Utzinger, S. Poppert and S. L. Becker (2021). "Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry." *Microorganisms* 9(1): 82.

Széll, Z., Z. Tolnai and T. Sréter (2013). "Environmental determinants of the spatial distribution of *Alaria alata* in Hungary." Veterinary Parasitology 198(1-2): 116-121.

Tăbăran, F., A. D. Sándor, M. Marinov, C. Cătoi and A. D. Mihalca (2013). "*Alaria alata* Infection in European mink." Emerging Infectious Diseases 19(9): 1547-1549.

Takata, I. (1951). "Experimental infection of man with *Ascaris* of man and the pig." Kitasato Archives of Experimental Medicine 23(4): 49-59.

Takeuchi-Storm, N., M. N. Al-Sabi, S. M. Thamsborg and H. L. Enemark (2015). "*Alaria alata* Mesocercariae among feral cats and badgers, Denmark." Emerging Infectious Diseases 21(10): 1872.

Taylor, M. H., P. O'connor, C. Keane, E. Mulvihill and C. Holland (1988). "The expanded spectrum of toxocaral disease." The Lancet 331(8587): 692-695.

Vieira, F. M., J. L. Luque and L. C. Muniz-Pereira (2008). "Checklist of helminth parasites in wild carnivore mammals from Brazil." Zootaxa 1721(1): 1-23.

Voelkel, A. C., S. Dolle, M. Koethe, J. Haas, G. Makrutzki, S. Birka, E. Lücker and A. Hamedy (2019). "Distribution of *Alaria* spp. mesocercariae in waterfrogs." Parasitology Research 118(2): 673-676.

Wang, C., C. Huang, P. Chan, P. Preston and P. Chau (1983). "Transverse myelitis associated with larva migrans: Finding of larva in cerebrospinal fluid." The Lancet 321(8321): 423.

Webster, P., C. Maddox-Hyttel, K. Nöckler, A. Malakauskas, J. Van der Giessen, E. Pozio, P. Boireau and C. M. O. Kapel (2006). "Meat inspection for *Trichinella* in pork, horsemeat and game within the EU: Available technology and its present implementation." Eurosurveillance 11(1): 15-16.

Wieser, A., L. Schneider, J. Jung and S. Schubert (2012). "MALDI-TOF MS in microbiological diagnostics – identification of microorganisms and beyond (mini review)." Appl Microbiol Biotechnol 93(3): 965-974.

Wolfe, A., S. Hogan, D. Maguire, C. Fitzpatrick, G. Mulcahy, L. Vaughan, D. Wall and T. Hayden (2001). "Red foxes (*Vulpes vulpes*) in Ireland as hosts for parasites of potential zoonotic and veterinary significance." Veterinary Record 149(25): 759-763.

Yong, T.-S., K.-J. Lee, M. H. Shin, H. S. Yu, U. Suvonkulov, T. B. Sergeevich, A. Shamsiev and G.-M. Park (2019). "Prevalence of Intestinal Helminth Infections in Dogs and Two Species of Wild Animals from Samarkand Region of Uzbekistan." The Korean Journal of Parasitology 57(5): 549-552.

Zimmermann, R., P. Combemale, M. A. Piens, M. Dupin and C. Le Coz (1995). "Cutaneous larva migrans, autochthonous in France. Apropos of a case." Annales de Dermatologie et de Venereologie 122(10): 711-714.

Publications and congress presentations

Peer-reviewed publications

2021 **Kästner C.**, Bahn P., Schönfelder R., Ozoliņa Z., Alksne L., Richter M.H., Deksne G., Mayer-Scholl A. and Johne A.
Development of a Novel Method for Identification of *Alaria alata* Mesocercariae by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.
Microorganisms **9**(8): 1664.
https://doi.org/10.3390/microorganisms9081664

2021 **Kästner C.**, Bier N.S., Mayer-Scholl A., Nöckler K., Richter M.H. and Johne A.
Prevalence of *Alaria alata* mesocercariae in wild boars from Brandenburg, Germany.
Parasitology Research (2021) 120:2103–2108.
https://doi.org/10.1007/s00436-021-07178-9

2020 Karadjian G., **Kaestner C.**, Laboutière L., Adicéam E., Wagner T., Johne A., Thomas M., Polack B., Mayer-Scholl A. and Vallée I.
A two-step morphology-PCR strategy for the identification of nematode larvae recovered from muscles after artificial digestion at meat inspection.
Parasitology Research (2020) 119:4113–4122.
https://doi.org/10.1007/s00436-020-06899-7

Oral presentations

2021 **Kästner C.**, Bahn P., Ozolina Z., Alksne L., Deksne G., Mayer-Scholl A., Johne A.
Identification of *Alaria alata* mesocercariae by MALDI-TOF mass spectrometry
13th European Multicolloquium of Parasitology, Belgrad, Serbien; 12.-16.10.2021

2019 **Kästner C.**, Mayer-Scholl A., Richter M.H., Johne A.
MALDI-TOF-Massenspektrometrie zur Identifizierung von *Alaria alata*
DVG-Tagung der Fachgruppe „Parasitologie und parasitäre Krankheiten“, Leipzig, Deutschland; 17.-19.06.2019

2019 **Kästner C.**, Mayer-Scholl A., Johne A.
MALDI-TOF mass spectrometry for identification of *Alaria alata*
Junior Scientist Zoonoses Meeting, Berlin, Deutschland; 20.-22.06.2019

Poster presentations

2019 **Kästner C.**, Mayer-Scholl A., Richter M.H., Johne A.
MALDI-TOF-Massenspektrometrie zur Identifizierung von *Alaria alata*
60. Arbeitstagung des Arbeitsgebietes Lebensmittelsicherheit und Verbraucherschutz, Garmisch-Partenkirchen, Deutschland; 24.-27.09.2019

2019 **Kästner C.**, Mayer-Scholl A., Richter M.H., Nöckler K., Johne A.
MALDI-TOF mass spectrometry for identification of *Alaria alata*
Junior Scientist Zoonoses Meeting, Berlin, Deutschland; 20.-22.06.2019

Danksagung

Zunächst möchte ich mich bei **Prof. Dr. Karsten Nöckler** und **Prof. Dr. Thomas Alter** für die offizielle Betreuung und Begutachtung dieser Arbeit bedanken.

Weiterhin gilt mein Dank **Dr. Martin Richter**, der mir über den gesamten Zeitraum der Promotion die Finanzierung dieser Arbeit ermöglicht hat. Ich danke Dir ganz herzlich für Deinen Einsatz und Dein Engagement, das ich immer sehr geschätzt habe.

Mein größter Dank gilt meinen Betreuerinnen **Dr. Annette Johne** und **Dr. Anne Mayer-Scholl** für die stetige Unterstützung und Hilfsbereitschaft sowohl bei der Methodenetablierung als auch beim Verfassen wissenschaftlicher Arbeiten. Ich danke Euch insbesondere für Euren immerwährenden Optimismus, die konstruktive Kritik sowie die vielen hilfreichen Ratschläge, die maßgeblich zum Gelingen dieser Arbeit beigetragen haben. Weiterhin danke ich Euch ganz herzlich dafür, dass Ihr mir die Teilnahme an diversen wissenschaftlichen Austauschprogrammen ermöglicht habt, die meine Promotionsjahre am Institut enorm bereichert haben.

Ein weiterer großer Dank gilt allen Kolleginnen und Kollegen der Fachgruppe 45 „Diagnostik, Erregercharakterisierung, Parasiten in Lebensmitteln“ für die unvergesslich schöne Zeit, die anhaltende Hilfsbereitschaft und Unterstützung in allen wissenschaftlichen und technischen Belangen.

Hervorheben möchte ich an dieser Stelle **Peter Bahn** und **Nicole vom Ort**, die mich insbesondere während der Entwicklung der MALDI-TOF-Methode immer mit großem Engagement unterstützt und beraten haben. Ich danke Euch für Eure Energie und Zeit, die Ihr in das Projekt investiert habt und bin sehr dankbar, dass ich Euch als Ansprechpartner hatte. Außerdem gilt mein Dank **Cornelia Göllner** für die engagierte Einarbeitung und Betreuung im Bereich der Molekularbiologie sowie **Nora Thaben** für die Bereitstellung von Probenmaterial und die Unterstützung bei der Probenbearbeitung.

Weiterhin danke ich **Kaya Stollberg**, **Susanne Diescher**, **Smita Sutrave**, **Nadja Bier** und **Enno Luge** für die vielen heiteren Momente und die stetige Unterstützung in allen Lebenslagen. Ohne Euch wären meine Promotionsjahre nur halb so schön gewesen – danke!

Zuletzt danke ich meiner Familie und meinen Freunden für die moralische Unterstützung und all die ermutigenden Worte und Ratschläge!

Angaben zu Finanzierung und Interessenskonflikten

Die Arbeiten wurden nicht finanziell unterstützt.

Es besteht kein Interessenskonflikt durch finanzielle Unterstützung der Arbeiten.

Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 06.05.2022

Carolyn Kästner

Druck: Mensch und Buch Verlag Berlin