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Sarcocystis rileyi (Apicomplexa) in Anas platyrhynchos in Europe with a potential for spread

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ABSTRACT

Four specimens of mallard (Anas platyrhynchos) shot by local hunters (December 2020 to January 2021 along the eastern coastline of the island of Bornholm in the Baltic Sea) were diagnosed with a heavy load of sarcocysts in the musculature. Morphometric and molecular diagnosis based on rDNA (18 S, ITS1, 28 S) of parasites recovered from two of the birds revealed the causative pathogen to be Sarcocystis rileyi. We further present novel sequences for the entire 5.8 S and ITS2 for this species. Elongate cysts (mean length 5.25 (SD 0.6) mm, width 1.37 (SD 0.2) mm) were recorded in all parts of the striated skeletal musculature of the birds. The main part (72%) of the 2585 cysts in one female mallard was located in the outer superficial pectoral musculature, with 11% in the inner pectoral musculature. Minor but significant parts were found in the dorsal, ventral abdominal, neck and head, legs, hand and arm (wing) musculature. No cysts were found in the smooth musculature. Each cyst contained a median of 3.2 mio bradyzoites indicating that more than 8 billion bradyzoites are available for infection of one or more predators/scavengers ingesting the bird. Bradyzoites (median length 13.5 μm (range 12.1–14.5) and median width 2.66 μm (range 2.1–3.3)) were highly resistant to proteinase treatment, which secures the passage through the stomach of the predator to its intestine where wall penetration takes place. One of the birds was ringed (tagged) in Sweden Island (land in the Baltic Sea two years before being shot. This is documenting immigration of mallards from northern locations. The parasite species was originally described in North America in 1893 and was commonly reported in this region during the 20th century but not in Europe. Recent cases from Norway, Finland, Lithuania, Poland, UK and Hungary suggest that the species may be spreading geographically.

1. Introduction

Sarcocysts are apicomplexan parasites using a two-host life-cycle. They are intracellular protozoans and infect the musculature forming cysts in muscle cells of mammals, birds and reptiles (acting as intermediate hosts). Numerous bradyzoites are produced within the sarcocyst and when a predator (the final host) ingests the infected animal the bradyzoites invade the intestinal wall, conduct sexual propagation and form oocysts leaving the predator along with feces. Oocysts are then infective to the intermediate hosts. Numerous species occur in farm animals such as cattle, sheep, goat, pig, horse (Deplazes et al., 2013) and poultry (Pan et al., 2020). The sarcocysts are difficult to identify to species level macroscopically but molecular tools (sequencing of ribosomal DNA and mtDNA) have proved invaluable for diagnosing. Using these techniques a series of new species have been described and investigations of wild bird populations indicate that a relatively high host specificity exists in several cases. Recent studies described infections with S. cornixi in hooded crow (Corvus cornix) (Kutkiene et al., 2009), S. tardus in blackbird Turdus merula (Kutkiene et al., 2012), S. columbae, S. halieti, S. lari and S. wobeseri in herring gull (L. argentatus) (Prakas et al., 2020b) and S. kutkenienae from common raven (Corvus corax) (Prakas et al., 2020a) while the sarcocysts in song thrushes await further identification (Cardells-Peris et al., 2020). Older North American studies showed that ducks, including mallard Anas platyrhynchos, host a
Sarcocystis species described as *S. rileyi* (Stiles, 1893). It has been regarded as common in North American birds, both in the nineteenth and twentieth century (Dubey et al. 2003), but European reports on sarcocysts in mallards have been rare. The sarcocysts are conspicuous and easily detectable and as mallard is a popular game bird it suggests absence or extreme rarity of sarcocysts in European mallards in the 20th century. However, in 2002 a Norwegian eider duck was found infected with sarcocysts (Gjerde, 2014) and in 2003 sarcocysts were reported in 1 out of 148 investigated mallards in Poland collected in 1999 and 2000 (Kalinska et al., 2003). The identification of sarcocysts in mallards to the species *S. rileyi* was later established for ducks in Finland and Lithuania (Prakas et al., 2014), UK (Cromie and Ellis, 2019; Muir et al. 2019) and in Hungary (Szekeres et al., 2019). The present study reports four cases of sarcocystosis caused by *S. rileyi* in mallards from the island of Bornholm in the Baltic Sea, a central location between Scandinavian and European countries and placed on the route of migrating birds between south and north. We present molecular data, distribution of sarcocysts in the muscle compartments, morphometric data of sarcocysts and bradyzoites, and histological examination of the infection site. We discuss the origin, possible introduction and probability of further spreading of the species in Europe.

### 2. Materials and methods

#### 2.1. Birds

Two local and experienced hunters on the island of Bornholm in the Baltic Sea reported presence of numerous white rice grain like particles. The findings were characterized as unprecedented by the hunters. Two of the birds in the pectoral musculature of 4 mallards shot during December 2020 were fixed in 4% neutral formalin (Hounisen Laboratory, Denmark) and prepared by homogenization of the individual sarcocyst in 10 ml water. The accuracy) for length and width. A suspension of bradyzoites was prepared by lysis of the bradyzoites 20 suspensions). One of the birds (a male) was previously ringed (Sweden, Stockholms, island) and H. The PCR outline was pre-denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, followed by annealing at an assay specific temperature (Table 1) for 30 s, and ending with elongation at 72 °C for 2 min and finally a post-elongation step at 72 °C for 7 min. The elongation time for the PCR was 3 min. For the lysis of the bradyzoites 20 suspensions (volume 1 µl) were taken and the number of bradyzoites enumerated whereafter the total number of bradyzoites per sarcocyst was calculated by multiplying with the dilution factor. A total of 50 samples were counted (5 from each of ten individual cyst suspensions).

#### 2.3. Morphometric and numerical measurements

A total of 50 cysts were measured using a Vernier gauge (0.1 mm accuracy) for length and width. A suspension of bradyzoites was prepared by homogenization of the individual sarcocyst in 10 ml water. The length and width of 50 bradyzoites in ten individual sarcocysts were measured microscopically (magnification ×400–1000) (accuracy 1 µm) (Leica DMBL, Leica Microsystems, Germany). From each sarcocyst suspension five subsamples (volume 1 µl) were taken and the number of bradyzoites enumerated whereafter the total number of bradyzoites per sarcocyst was calculated by multiplying with the dilution factor. A total of 50 samples were counted (5 from each of ten individual cyst suspensions).

#### 2.4. Whole mount

Isolated sarcocysts were liberated from host material, rinsed in water and fixed in 4% neutral formalin for 24 h. They were then cleared in lactic acid and embedded on microscope slides in Aquatex (Merck, Darmstadt, Germany).

#### 2.5. Histology

Sections of the pectoral musculature, containing sarcocysts, from the male mallard were fixed in 4% neutral formalin (Hounisen Laboratorium, Skanderborg, Denmark) for 24 h, transferred to 70% ethanol and dehydrated in graded ethanol series until two shifts of Shondon Xylene Substitute (Fisher Scientific, Denmark) whereafter the specimen was embedded in paraffin. Sections of 5 µm were prepared and stained by haematoxylin and embedded in DePeX mounting medium (Sigma-Aldrich, Denmark). Sections were studied in a Leica DMBL light microscope and micrograph pictures recovered by a Leica CD300 (Leica Microsystems, Germany).

#### 2.6. Molecular analysis

Six individually isolated sarcocysts (three from each of the two birds) were subjected to lysis, DNA-purification, PCR with specific primers (Table 1), product purification, sequencing and BLAST analysis. Genomic DNA from each individual sarcocyst was extracted by use of a QIAGEN® DNeasy Blood & Tissue Kits according to the manufacturer’s recommendation. PCR was performed in 60 µl PCR mix which contain 2 µl sample 6 µl forward primer (10 mM), 6 µl reverse primer (10 mM), 6 µl 10× PCR-buffer, 6 µl dNTP (4 × 10 mM), 1.8 µl MgCl2 (50 mM), 0.25 Polymerase (BIOTAQ DNA Polymerase, Saveen & Werner ApS, Denmark) and H2O. The PCR outline was pre-denaturation at 95 °C for 5 min; amplification and H2O. The PCR outline was pre-denaturation at 95 °C for 5 min; amplification and 270 °C for 2 min and finally a post-elongation step at 72 °C for 7 min. The elongation time for the PCR using BD1 vs KL-P1R was 3 min. For the lysis of the bradyzoites 20 µl of

### Table 1

| Region | Primer | Purpose | Primer sequence (5′→3′) | Ta | Product length | Reference |
|--------|--------|---------|-------------------------|----|----------------|----------|
| 18 S   | SarAF  | PCR & Seq. | cgggttgatcctgcgcaagt | 51 °C | 960 bp | Kutkien et al. (2010) |
|        | SarBR  | PCR & Seq. | ggenaatggctctgagtag | 51 °C | 895 bp | Kutkien et al. (2010) |
|        | SarCF  | PCR & Seq. | tttaactgtgagaggaattct | 51 °C | 1197 bp | (Gjerde 2014) |
|        | SarDR  | PCR & Seq. | ggataagttcaacggaagaa | 53 °C | 3024 bp | Galazo et al. (2002) |
|        | SU1F   | PCR & Seq. | gcattgaggttgcaggaatttt | 53 °C | 1568 bp | Kutkien et al. (2010) |
|        | 5.8R2  | PCR & Seq. | aaaggctatgattgcttgaagaa | 53 °C | 1568 bp | Kutkien et al. (2010) |
| 18 S   | ITS1   | PCR & Seq. | ggtctgagttcaacggaagaa | 53 °C | 3024 bp | Galazo et al. (2002) |
|        | ITS1.5-8 S-ITS2-28 S | PCR & Seq. | cccaaagttgggaagagatt | 53 °C | 1568 bp | Kutkien et al. (2010) |
| 28 S   | PTN#390 R | Seq. | gatccgtggttaagcggg | 51 °C | 960 bp | Kutkien et al. (2010) |
|        | KL-P1F | PCR & Seq. | tgcctgcttgtcagacat | 51 °C | 960 bp | Kutkien et al. (2010) |
|        | KL-P2R | PCR & Seq. | ggcgtgcgtgtcagacat | 51 °C | 960 bp | Kutkien et al. (2010) |
the Proteinase K solution (>600 mAU/mL) was used in 200 μl lysis reactions, whereby the concentration of Proteinase K was >60 mAU/mL. The lysis had to be prolonged, according to the manufacturer’s manual, to 24 h because bradyzoites were found intact (microscopic examination) after 5 h incubation. PCR products purified using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (cat.no. 28-9034-71,VWR, Denmark) were sequenced at Macrogen Europe B.V., Netherlands. All six purified products were subjected to individual sequencing and subsequent analyses. Thus, six independent alignments of sequences were obtained and analyzed individually.

3. Results

3.1. Infection data

The two mallards, of which at least one of the birds had immigrated from Sweden, as documented by the ring data, were found heavily infected with cysts of *Sarcocystis rileyi* throughout their body musculature (Fig. 1). A total of 2585 sarcocysts were recovered from a single mallard. The main part of the cysts and the bradyzoites within them were located in the superficial pectoral muscles (accounting for 72% of all cysts) but also the back, ventral abdomen, neck, head, leg and wing (arm/hand) muscles were infected (Table 2).

3.2. Morphometric data

The sarcocysts were rice grain shaped (Fig. 2A) and contained numerous bradyzoites, which we released by homogenization in water (Fig. 2B). The histology showed the thin cyst wall surrounding an ordered system of bradyzoites (Fig. 2C and D). The sarcocysts were surrounded by a single cyst wall and in some cases, but not all, encapsulated by a collagenous amorphous layer in the striated muscle tissue. Some sections revealed accumulation of host cells (Fig. 2C and D). The bradyzoites in the intact sarcocyst were densely packed and the nuclei heavily stained (Fig. 2D). In addition, the morphometric data of sarcocysts and bradyzoites (Table 3) complied with the species descriptions provided by Dubey et al. (2003). The mean size of the cysts was 5.25 (SD 0.6) mm in length and the width 1.37 (SD 0.20) mm. The elongated bradyzoites with a subterminally located nucleus had a median length of 13.5 (range 12.1–14.5) μm and a median width of 2.6 (range 2.1–3.3) μm. Each of the cysts contained a median number of 3.2 mio bradyzoites (range 1.9–5.0 mio) and with a total of 2585 cysts in one single host, the bird represents an infective potential of more than 8 billion bradyzoites.

3.3. Sequence data

PCR studies were conducted individually for six isolated sarcocysts. Performing five PCR resulted in overlapping products (GenBank accession nos. MZ468637 to MZ468641). These products may be combined into a single sequence 5349 bp long comprising the partial 18 S, the

![Fig. 1. Sarcocystis rileyi sarcocysts in mallard musculature. A. Pectoral musculature, B. Dorsal musculature, C. Hand and arm musculature, D. Leg musculature.](image_url)
complete ITS1, the complete 5.8 S, the complete ITS2 and partial 28 S. All six analyzed sarcocysts displayed the same sequences. Fig. 3 presents an alignment of the obtained sequences of the five PCRs together with 100% similar sequences at GenBank (See Table 1 for obtained length of PCR products, annotated using the internet resource Rfam). The molecular diagnosis (Table 4) gave a precise designation with a high ITS sequence similarity to parasites recovered in the USA, Norway & UK (Table 4) supported by a high 18 S similarity to parasites recovered in Norway, UK and Lithuania and by a high 28 S similarity to parasites recovered in Norway, Lithuania and USA. Only 60 ITS2 sequences of the family Sarcocystidae (designated *Sarcocystis* sp. with accession numbers MH590230 & MH590233) (Lee, 2019) representing seven species were available at GenBank. Despite the high quality of sequence data from the present work (PCR product using BD1 vs KL-P1R), the ITS2 of *S. rileyi* had no obvious similarity to any ITS sequence at GenBank. Using the least stringent BLAST option (“Somewhat similar sequences”), the only hit at Genbank was a 73 bp long fragment with no annotation from the *Rhinatrema bivittatum* (two-lined caecilian) chromosome 12 (LR584398) with 78% similarity towards the obtained ITS2. The 5′ end and the 3′ end of this sequence of the PCR product overlapped the GenBank *S. rileyi* sequences KJ396584 and HM185743 with 1070 bp and 1004 bp, respectively. The ITS2 of *S. rileyi* is 285 bp and 321 bp longer than the reported *Sarcocystis* sp. sequences. The resulting 5349 bp long sequence of *S. rileyi* has been uploaded to GenBank (accession number MZ151434) which represent the first report of the complete ITS2 of *S. rileyi*.

4. Discussion

The two mallards obtained from local recreational hunters on the island of Bornholm in the central Baltic Sea were found heavily infected with cysts of *Sarcocystis rileyi* throughout their body musculature. The main part of the cysts were located in the superficial pectoral muscles but also the dorsal, leg and arm muscles were highly infected and thereby infective to a predator or scavenger. The molecular diagnosis gave a precise designation with a high ITS1 sequence similarity to the species records in the USA, Norway, UK, Finland and Lithuania (Gjerde, 2014; Muir et al. 2019; Prakas et al., 2020). By combining various primer sets and analyzing overlaps we were able to present novel sequences for rDNA segments 5.8 S and ITS2 for *S. rileyi*. In addition, the morphometric data complied with the species descriptions provided by Dubey et al. (2003). At least one of the infected birds had immigrated from Sweden, as documented by the ring data, and it shows that the distribution area of a certain avian parasite species can be expanded easily with bird migration. In this context it should be considered that mallard migration and distribution entails a potential for fast and wide dispersal of parasites easily covering the whole Baltic sea (van Toor et al., 2018). During the 20th century reports on occurrence of sarcocysts in European mallards were limited whereas North America appeared as the main area of distribution of sarcocyst infected birds. Sarcocysts were observed in North American birds by Riley in 1869 and *S. rileyi* was then described by Stiles in 1893, whereafter numerous studies through the 20th century documented the species in American ducks (Dubey et al., 2003). The occurrence of a sarcocyst infected mallard in Poland 1999) to 2000 (finding one infected bird out of 148 examined) (Kalisinska et al., 2003) and one infected eider duck in Northern Norway in 2002 (Gjerde, 2014) were considered unusual findings. The parasite was later recorded from Finland and Lithuania (18 and 12 birds, respectively, found infected in...
the period 2011–2013) (Prakas et al., 2014), UK (90 reports 2015–2017) (Cromie and Ellis, 2019; Muir et al., 2019), Hungary (12 infections reported, identification of S. rileyi in two birds) (Szekeres et al., 2019) and from Denmark (Baltic region) (4 infected out of more than 93 ducks examined) (present study). This is the first record of S. rileyi in Denmark and the finding, characterized by local hunters as highly unusual and unprecedented, suggests that the species has a potential for spread in Europe. It may be discussed if the occurrence has been underreported in Europe (Szekeres et al., 2019; Muir et al., 2019) but a local hunter from the island of Bornholm, with 40 years experience and always having a habit of removing skin of the duck, claimed that he had never seen such an infection before. Alternatively, the parasite could have been present at very low prevalence in Europe and due to changes of one or more environmental factors (abiotic or biotic) the transmission, and thereby the prevalence in mallards, would rise.

An introduced parasite species with a complicated two-host life cycle may become established in a certain geographic region depending on the presence of suitable hosts. The birds act as intermediate hosts and final host species recorded for S. rileyi are skunk (Wicht 1981; Dubey et al., 2003), fox and raccoon dog (Prakas et al., 2015; More et al., 2016; Szekeres et al., 2019). Further, it was suggested by Gjerde (2014) that apart from canids, such as polar fox, also American mink (Mustela vison) and stout (Mustela erminea) could serve as final hosts. European countries are populated by foxes, raccoon dogs, stout and naturalized American mink, whereby the potential for further spread in European mallard populations is present. Even on the island of Bornholm (where fox, raccoon dog and stout are absent) the possibility for establishment is present due to a significant population of American mink. Our histological sections showed that the bradyzoites in the sarcocysts are densely packed but when the cysts are homogenized in water the elongate cells are stretched. The resistance to Proteinase K treatment indicates that the bradyzoites are able to resist the enzymes in the predator’s stomach and intestine following ingestion.

No controlled pathogenicity studies in birds infected by S. rileyi are available but histopathological studies have shown various (from light to severe) forms of necrosis and inflammation in the muscles of infected birds (Muir et al., 2019). The heavy infections in the entire musculature in the present study suggests that flying ability may be depressed in these birds. Severe disease signs, including muscular malfunctions, may appear in chickens infected with other species of Sarcocystis (Munday et al. 1977; Muralib et al. 1995), a situation known from infections in other animals acting as intermediate hosts (Deplazes et al., 2013). It cannot be excluded that S. rileyi could spread further and impact European duck populations. It is therefore recommended that European bird populations become surveyed and sarcocyst infections recorded.

5. Conclusion

Infections with S. rileyi in mallard A. platyrhynchos have been prevalent in North America but probably absent or very rare in Europe during the 20th century. Since turn of the millennium an increasing

| Region | Aligned Length | GenBank acc. No. | Host | Country | Percentage identity |
|--------|----------------|-----------------|------|---------|---------------------|
| 18 S   | 1784           | KJ396583        | Somateria mollissima | Norway | 100 |
|        |                 | LT992317        | Anas platyrhynchos  | United Kingdom | 100 |
|        |                 | HM185742        | Anas platyrhynchos  | Lithuania | 100 |
|        | 937            | LT992314        | Anas platyrhynchos  | United Kingdom | 100 |
| ITS1   |                 | KJ396584        | Somateria mollissima | Norway | 100 |
|        |                 | GU188427        | Anas platyrhynchos  | USA: Colorado | 99 |
| 28 S   | 1525           | GU188426        | Anas platyrhynchos  | USA: Colorado | 100 |
|        |                 | HM185743        | Anas platyrhynchos  | Lithuania | 100 |
|        |                 | KJ396585        | Somateria mollissima | Norway | 99 |

Table 4

Sequences for Sarcocystis rileyi obtained from two mallards (6 identical sequences from 6 sarcocysts) shot on the island of Bornholm (Baltic Sea) aligned with GenBank sequences for S. rileyi in North America, UK, Norway and Lithuania. PCR primer binding sites were excluded from the sequences before comparison. The ITS2 of S. rileyi is exclude from this table because it exhibited no obvious similarity to any ITS sequence at GenBank.
number of cases have been reported from Europe and it cannot be excluded that the parasite species is spreading in European countries. The severe infection affecting the entire striated body musculature and with a major sarcocyst occurrence in the flying apparatus (pectoral muscle, wings) suggests a pathogenic effect on the host. Pathogenicity studies should be implemented and surveys for monitoring the infection status in European bird populations should be prioritized.

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**Declaration of competing interest**

The authors declare that they have no conflicts of interests.

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