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Article

Cardiac filariosis in migratory Mute swans (Cygnus olor) in Sicily

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ABSTRACT

Sarconema eurycerca is a common parasitic disease of North America swans and geese. The infection has been correlated with severe heart lesions, often resulting in cardiac failure and death of the animals. Heartworms infections have been previously reported in European swans, and specifically in the United Kingdom and Nederland. Both the countries are characterized by a cold temperate weather, similar to the one that can be found in swan wintering areas of U.S.A. and Canada. The first record of cardiac filariasis associated with Sarconema eurycerca infection in four swans in Italy. Twelve mute swans were examined during avian influenza surveillance activities on migratory birds. Birds were collected in the year 2006, in wintering areas of Eastern Sicily (Italy). Four of the twelve swans showed necrotic-haemorrhagic myocarditis with intra-lesional nematodes. Morphological characteristics identified the parasite as a filarial nematode. Birds lungs samples were used for parasites DNA extraction. The latter was used as template for polymerase chain reaction (PCR) amplification and sequencing of part of the 12S rDNA gene. Comparison of genomic DNA extracted from a reference S. eurycerca isolate confirmed parasite identity and provided the first sequence resources for this species of value to future diagnostic and epidemiological studies

1 Introduction

Heartworm disease in swans and geese is caused by *Sarconema eurycerca*, a filarial nematode (Spirurida, Filarioidea, Onchocercidae, Lemdaninae) transmitted between birds by the biting louse *Trinoton anserinum* (Cohen *et al.*, 1991). Studies with *S. eurycerca* have shown it to be pathogenic to some geese and swan species including whistling swans (Cygnus columbianus), trumpeter swans (Cygnus buccinators), Canada geese (Branta Canadensis) and white-fronted geese (Anser albifrons) (Holden and Sladen, 1968; Kluge, 1967; MacNeil, 1975). In these species massive infestation with *S. eurycerca* have been related to severe heart lesions resulting in cardiac failure and death (Kluge, 1967; Cole, 2013). Mild infestation may not be pathogenic (Holden and Sladen, 1968) and the parasite may infect and develop within the bird with no clinical evidence (Cole, 2013). Disease caused by *S. eurycerca* is common in North America (U.S.A. and Canada). To the authors knowledge the available literature pointed out the presence of *S. eurycerca* only in Nederland and United Kingdom, among all the Europeans countries (Cohen *et al.*, 1991; de Bruijn *et al.*, 2009).

S. eurycerca has an indirect life cycle that includes three larval stages. The larvae develop in an intermediate host (a louse) prior to infecting its definitive host (birds), where they become adult and reproduce. Adult female heartworms release microfilariae into the blood stream of the definitive host bird. The microfilariae subsequently infect the biting louse *T. anserinum* during feeding upon the bird. A new host bird becomes infected when the louse bites it to feed on its blood and infective larvae move into the bird's bloodstream. The larvae commonly migrate to the myocardium, develop to sexual maturity and release microfilariae into the blood stream (Cole, 2013).

The incidence of *S. eurycerca* in Whistling swans in the Western lakes area of the United States reported by Quortup and Holt (Quortup & Holt, 1940) was 18.5%, similar to the incidence of 16.2% reported in a field survey carried out by Holden and Sladen (Holden and Sladen, 1968). Cole (Cole, 2013) observed variable prevalence (4-20%) based upon microscopic examination of blood smears taken from apparently healthy birds. The occurrence of *Sarconema sp*. in Canadian swans is rarely reported (MacNeil, 1975). To our knowledge few data are available on pathology related to *Sarconema sp.* infestation in European swans (de Bruijn *et al.*, 2009; Oğuz *et al.*, 2015) and currently no sequence data has been made publically available, hindering molecular identification and diagnosis.

2 Material and Methods

Case history. In wintering areas of migratory birds of Eastern Sicily (Italy), in early February 2006 during surveillance activities against Avian influenza (AI; as established by Italian regulations, Ministry of Health n. 40129 dated November 11th 2005), twelve clinically suspected swans were trapped and humanely euthanized. The swans belonged a group of 19 birds where previously n. 8 were found to be positive for the pathogenic H5N1 strain.

Gross findings and histopathology. All swans were examined by a full necropsy. Different tissues samples (lungs, heart, kidney, intestine, spleen, liver and brain) were collected from each bird for detection of AI virus and other differential laboratory investigations including histopathology.

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Four µm thick sections were obtained by formalin-fixed paraffin-embedded. The preparations obtained were dried overnight in an oven at 37 °C. It was proceeded with dewaxing by xylene for 20 min. After a descending alcohol series (100°, 95°, 75° and 50°), slides were washed in distilled water and then stained with haematoxylin and eosin. This was followed by the ascending scale of alcohols (50°, 75°, 95° and 100°) and clarification in xylene. After this phase, the slides were mounted in Acrylic mounting medium (Eukitt®, O. Kindler GmbH).

Detection of AI virus. Samples of the same organs collected for histology were used for quantitative real time polymerase chain reactions (qRT-PCR) for the gene M of the avian influenza virus as described elsewhere (Spackman *et al.*, 2002).

2.1 Identification of parasites.

Histology. Histological identification of parasites found in the lesions was performed according to published data (Gardiner & Poynton, 2006).

Polymerase Chain Reaction (PCR) and sequencing. DNA samples were extracted from formalin fixed, paraffin embedded tissues from each infected animal. Four 10 µm sections for each specimen were pre-treated according to the DNeasy Blood & Tissue Kit instructions as recommended by the manufacturer (Qiagen GmbH; Hilden Germany) for formalin-fixed tissues. The DNA extraction followed the manufacturer's instructions for total DNA purification from animal tissues. 12S rDNA amplifications and sequences were obtained following Casiraghi *et al.*, (2004). In particular, DNA sequences were amplified using the forward primer 12SF (5'-GTTCCAGAATAATCGGCTA-3') and the degenerate reverse primer 12SR (5'-ATTGACGGATG(AG)TTTGTACC-3'). PCRs were performed in 20 µl volumes under the following conditions: 1x buffer, 1.5 mM $MgCl_2$ (Master Taq kit, Eppendorf™, Hamburg, Germany), 0.2 mM of each dNTP, 1 µM of each primer, and 1 U of polymerase (Master Taq kit, Eppendorf™, Hamburg, Germany). The thermal profile was the following: 94°C 45 sec, 50°C 45 sec, and 72°C 90 sec for 40 cycles. PCR products were gel purified (using the Perfect Prep Gel Clean-up, Eppendorf™, Hamburg, Germany) and directly sequenced using ABI technology. The four 12S rDNA sequences were aligned with ClustalX version 1.81 (Thompson *et al.*, 1997), manually curated and visualized with BioEdit (Hall, 1999).

A BLASTn analysis was performed using the National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov/blast) using default parameters and 'genomes: other', to identify similar annotated sequences. In parallel, genomic DNA was extracted from a reference S. eurycerca sample collected previously from a swan during post-mortem in January 1981 in Berkshire, UK, and identified by the Commonwealth Institute of Helminthology, St Albans, UK, under the reference 3608 to provide a valid comparison.

2.2 Phylogenetic comparison

Sequences found by BLASTn to be most similar to the 12S rDNA sequences generated here were downloaded from GenBank and used for phylogenetic comparison with Dirofilaria immitis, a filarial heartworm, included as an out group. The optimal models for PhyML and

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MrBayes phylogenetic analyses were selected using TOPALi v2.5 (Milne *et al.*, 2009), identifying TVM plus gamma (G) and GTR + G models respectively based on the Akaike Bayesian information criteria (AIC and BIC). A PhyML phylogeny was constructed using 1,000 bootstrap replications. A Bayesian phylogeny was constructed using 10,000,000 generations (10% discarded as burn-in) and sampled every 500. Two replicate analyses were performed to ensure convergence and the results were then pooled. MEGA v5.1 (Tamura *et al.*, 2011) was used to construct a Neighbor Joining phylogeny with the Kimura-2 parameter and 1,000 bootstrap replicates.

3 Results

Gross findings. At necropsy, all swans found to be were in good body condition with thick subcutaneous and cavitary fat, and no external lesions. In four swans multiple, scattered, small (1-3 mm) yellowish-tan, myocardial (necrotic-haemorrhagic) foci were detected.

Quantitative real-time PCR for AI virus. All birds were negative for AI virus.

Histopathology. Scattered heterophilic and macrophagic aggregates were observed in association with adult nematodes in the cardiac sections. Adult nematodes observed in cardiac lesions were approximately 600-800µm in diameter, with a 2-3µm cuticle, pseudocoelom and coelomyarian muscular layer (figure 1). Several microfilariae were detected in the distended uterus of adult female nematodes (figure 1). There were a few mineralization foci adjacent to the parasites (figure 1, inset). Curvilinear nematode parasites (about 100µm in length) with discernable nuclear columns consistent with microfilarial forms were detected in the myocardium parenchyma and in the cardiac blood vessels (figure 2). Interstitial fibrosis and degenerative changes were also observed associated with multiple haemorrhagic foci with deposits of hemosiderin (figure 2). Scattered necrotizing vasculitis (fibrinoid necrosis) with infiltration of heterophils and macrophages were observed (figure 3). No remarkable changes were detected in the other organs.

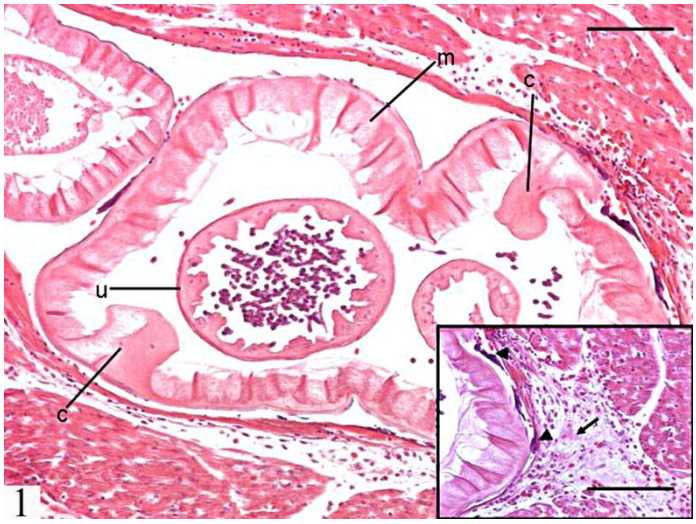

Figure 1. *Sarconema eurycerca* in the myocardium of a swan. Note the latheral chords (c), the coelomyarian musculature (m) and the uteri (u) contain numerous microfilariae. Haematoxylin and Eosin, bar = 35µm. Inset: the adult nematode is surrounded by mild inflammatory reaction, fibrosis (arrow) and mineral deposits (arrowheads). Haematoxylin and Eosin, bar = 35 µm.

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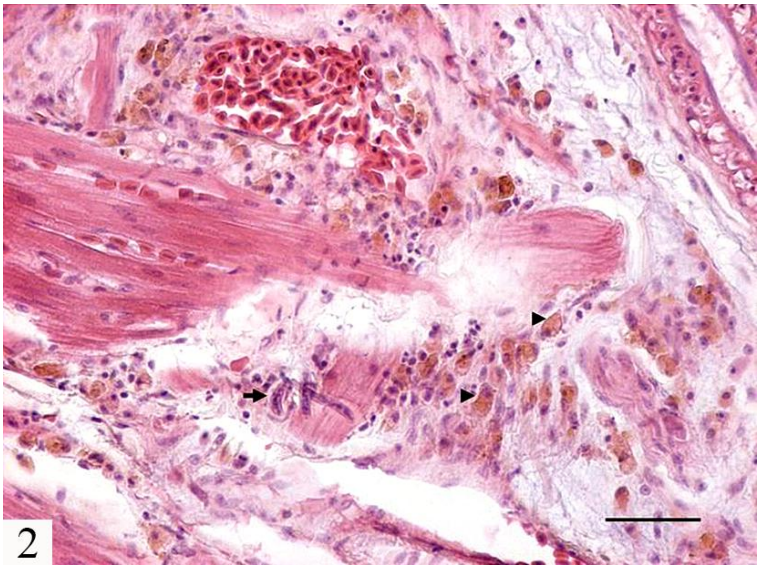

Figure 2. Microfilariae in the myocardium (arrow), degenerative changes of myocardial cells and focal hemorrhages contained siderophages (arrowheads). Haematoxylin and Eosin, bar = 50 µm.

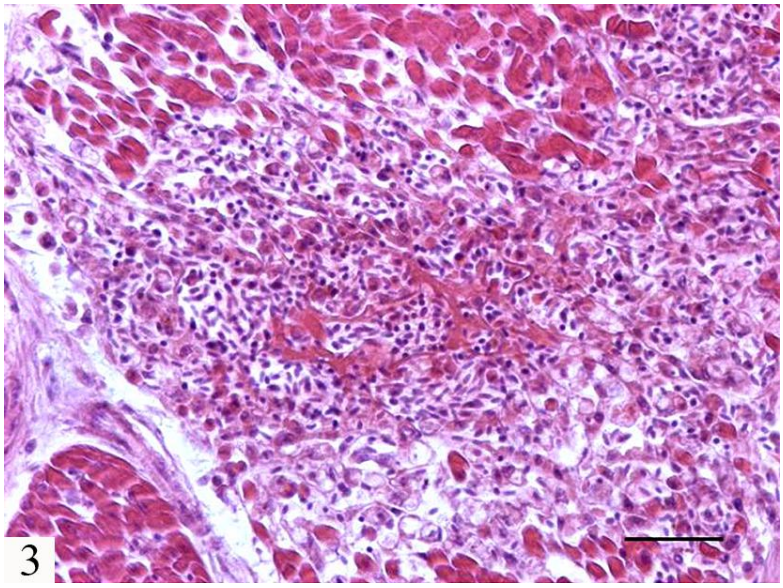

Figure 3. Necrotizing vasculitis (fibrinoid necrosis). Haematoxylin and Eosin, bar = 50 µm.

3.1 Parasite identification.

Histology. Morphologic characteristics of the parasites observed in cardiac lesions included: 1) presence of microfilariae within the adult females; 2) coelomyarian musculature; 3) lateral chords; 4) small intestine; 5) evenly spaced, external longitudinal cuticular ridges. These morphological characteristics identified the parasite as a filarial nematode (Gardiner and Poynton, 2006), with features similar to those described for Sarconema sp., possibly *S. eurycerca* (Kluge, 1967).

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PCR and sequencing. Sequencing the 12S rDNA amplified from genomic DNA purified from paraffin embedded tissues of four specimens (Cygnus olor) yielded four identical 483 bp sequences (100% identity, 100% similarity, BLASTn pairwise alignment). The sequence has been submitted to the EMBL Data Library according to the EBI Procedure under the accession number AM932375. Comparison with the equivalent 483 bp amplicon derived from a reference *S. eurycerca* sample revealed 100% sequence coverage with 481/483 identical nucleotide matches (accession number LN812979). The next closest matches were the 12S small subunit ribosomal RNA coding sequences from *Ascaridia galli* (JX624728) and *Ascaridia columbae* (JX624729), with 86%/84% similarity and 100%/85% query coverage respectively. The absence of single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) between the four 12S rDNA sequences indicate that no error was introduced by PCR or sequencing, and confirms that all the nematodes examined were the same species. BLAST comparison with the reference isolate sequence provided molecular confirmation of the histological identification as *S. eurycerca*. It is noteworthy that our submissions represent the first entries for the subfamily Lemdaninae.

Following BLASTn comparison eight additional nematode 12S rDNA sequences, including *D. immitis* as an outgroup, were downloaded and used for phylogenetic comparison with maximum likelihood (PhyML), Bayesian (MrBayes) and Neighbor Joining (NJ) approaches (figure 4). The reference and candidate *S. eurycerca* were most closely related, supporting our sample identification.

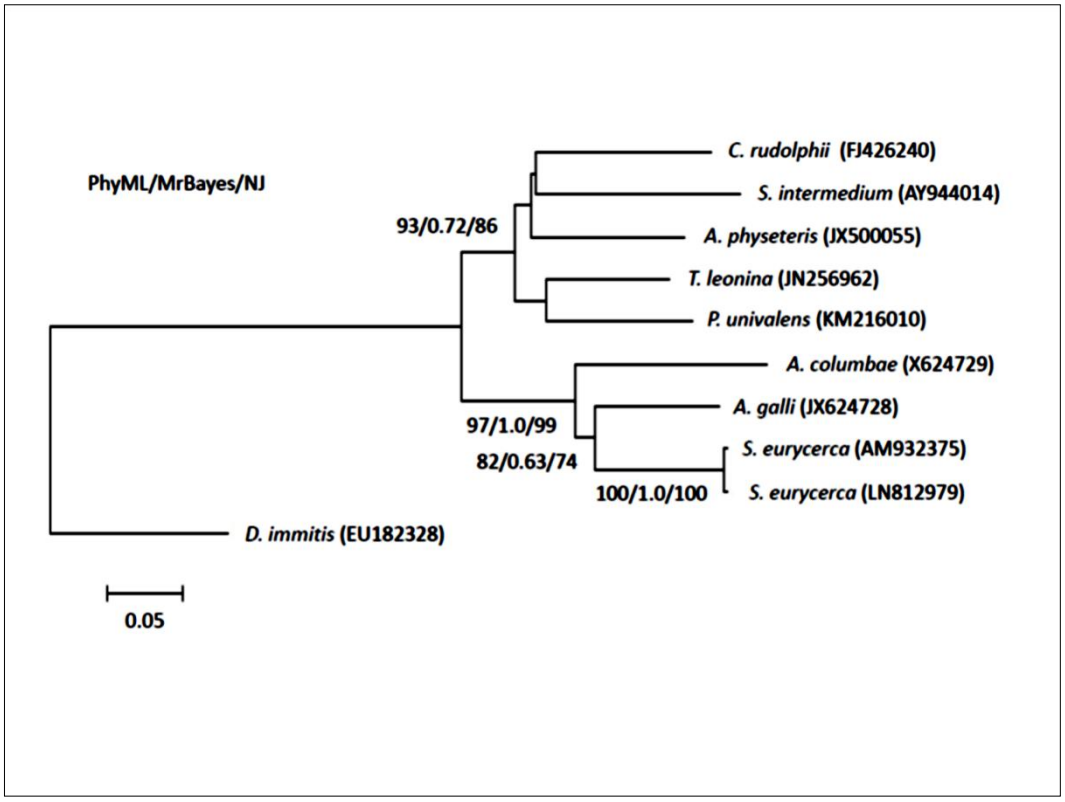

Figure 4. Phylogenetic comparison of 12S rDNA coding sequences representing *S. eurycerca* with *Anisakis_physeteris, Ascaridia columbae, Ascaridia galli, Contracaecum rudolphii, Parascaris univalens, Steinernema intermedium, Toxascaris leonina* and *Dirofilaria immitis* as an out group (accession numbers as shown). Branch values in excess of 70/0.7 are shown for Maximum Likelihood, Bayesian and Neighbor Joining models.

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4 Discussion

Swans filariosis caused by *S. eurycerca* has been documented in North America, Europe and Asia. (Holden and Sladen, 1968; Kluge, 1967; MacNeil, 1975; Oğuz *et al.*, 2015; Woo *et al.*, 2010) In Europe, only few cases have been reported in Nederland (de Bruijn *et al.*, 2009), Turkey (Oğuz *et al.*, 2015) and United Kingdom. (Cohen *et al.*, 1991) Here, the presence of cardiac lesions associated with *S. eurycerca* confirmed the pathogenicity of filariosis in swans. The finding of necrotizing vasculitis suggests an immune complex-mediated (type III) hypersensitivity pathogenic mechanism. Furthermore, this is the first report of *S. eurycerca* in Italy.

The first reports of these parasites are recorded in boreal to warm-temperate areas such as North America and Japan (Cohen *et al.*, 1991; MacNeil, 1975; Woo *et al.*, 2010). More recently the parasite has also been identified in warm temperate to sub-topical areas like Turkey. (Oğuz *et al.*, 2015) The spreading of the parasite is suggesting that global climate changes may have played a role in the diffusion of the parasites and/or the intermediate host. Nevertheless, the diffusion of these parasites can represent both a potential risk for domestic species and a risk for the European swan and geese population health. In domestic fowls preventions strategy should be practiced to avoiding the infestation. The use of proper restraining systems preventing direct contact between farmed and wildlife animals, and periodical treatment with insecticides should reduce the risk of diffusion of the intermediate host.

To our knowledge, there are no data in the literature about the prevalence of *Sarconema sp.* in Italian swans. In this record cardiac filariosis was detected in 4 (33%) swans out 12. Nevertheless, a larger population of swans should be examined, possibly from other Italian or European wintering regions, to determine the prevalence of filariosis in European swans. In Italy the warning and the emotional impact due to avian influenza risk and the outbreaks registered in wild species caused by virus H5N1-HPAI (High Pathogenicity Strain Avian Influenza), has stressed the importance of knowledge on ecology and pathology of migratory species and particularly of mute swan (*Cygnus olor*). This specie showed a primary role in avian influenza epidemic in Europe (Terregino *et al.*, 2006). This experience underlined the importance of migrating species and their relevance in diffusion of high risk zoonosis and moreover, it has showed interesting data on less known parasitic diseases of birds which can potentially represent a potential risk also for domestic species. Building on this we have established the first molecular sequence marker for *S. eurycerca*, providing a resource for the future identification of this parasite and discrimination from other closely related parasites.

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