Parasitology

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Pathology of *Angiostrongylus cantonensis* infection in two model avian hosts

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Research Article

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Abstract

Angiostrongylus cantonensis causes severe neurological disorders in a wide range of warm-blooded animals, including several avian species. A laboratory isolate of *A. cantonensis* originating from French Polynesia, genotyped as clade 2, was used to assess the effect of experimental infection in chicken and Japanese quail. Low dose groups of birds were infected orally by 100 L3 larvae, high dose groups by 1500 L3 larvae and the birds in the third group were fed three infected snails, mimicking a natural infection. Clinical signs during the first week after infection, haematology, biochemistry, gross lesions and histology findings were used to assess the pathology of the infection. Some of the infected birds showed peripheral eosinophilia, while mild neurological signs were seen in others. No larvae were observed in serial sections of the central nervous system of infected birds 1 week after infection and no major gross lesions were observed during necropsy; histopathology did not reveal lesions directly attributable to *A. cantonensis* infection. Our results suggest that galliform birds are not highly susceptible to *A. cantonensis* infection and open a question of the importance of Galliformes in endemic areas as natural pest control, lowering the number of hosts carrying the infective larvae.

Introduction

Angiostrongylus cantonensis affects a range of hosts in its circulation in the ecosystems. While the typical life-cycle with rats as definitive hosts and gastropods as intermediate hosts is well-studied (Bhaibulaya, 1975), most of the information available on other types of host–parasite interactions originate either from case reports or limited experimental evidence.

Poikilothermic vertebrates, such as reptiles and amphibians (Ash, 1968; Cowie, 2013), are usually considered paratenic hosts, which harbour infective L3 larvae in tissues. In homoeothermic vertebrates, however, after a short period of intravascular migration through the body, the larvae actively seek a way to the central nervous system (CNS), where they attempt to continue their development (Barratt *et al.*, 2016). While in some murine rodents the young adult nematodes migrate to the lungs and reach maturity, other mammals and birds are considered aberrant hosts, where dying *A. cantonensis* larvae in various stages of their development, together with the immune reaction of the host, affect the function of the CNS, peripheral nerves, lungs or other organs (Jindrak and Magnusson, 1981; Barratt *et al.*, 2016).

Human infections caused by *A. cantonensis* are well studied, with a variable symptomatology. While many severe and fatal cases are described in detail (Barratt *et al.*, 2016), mild infections probably remain underdiagnosed in many areas of the world (Sawanyawisuth and Chotmongkol, 2013). Domestic dogs represent another example of a highly susceptible aberrant host (Walker *et al.*, 2015) with over hundred cases of natural *A. cantonensis* infection described to date (Mason, 1987, 1989; Lunn *et al.*, 2003, 2012). Some avian species, like tawny frogmouths *Podargus strigoides* (Latham, 1801) and cockatoos, have been diagnosed as fatal cases of neural angiostrongyliasis (Monks *et al.*, 2005; Gelis *et al.*, 2011; Reece *et al.*, 2013).

Given the apparent pathogenicity of *A. cantonensis* infection for some avian species, the occurrence of potential intermediate and paratenic hosts in the diet of galliform birds and lack of experimental studies, we aimed to: (i) investigate the susceptibility of domestic chicken and Japanese quail to *A. cantonensis* infection, (ii) investigate the presence of larvae in the CNS and describe the clinical course of the early infection stage, (iii) assess the relationship between larval dose and pathology.

Materials and methods

Angiostrongylus cantonensis strain and experimental animals' origin

The experimental strain of *Angiostrongylus cantonensis* originates from Fatu Hiva, French Polynesia and has been kept in laboratory conditions since 2017, by circulating among

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Wistar laboratory rats and experimental water snails *Biomphalaria glabrata* (Say, 1818) and land snails *Subulina octona* (Bruguière, 1789) as intermediate hosts, to retain the infectivity of the strain. Identity of the *A. cantonensis* isolate was confirmed based on the morphology of adult nematodes from infected rats as well as by whole cytochrome c oxidase subunit 1 gene sequencing and the strain has been identified as part of the Clade 2 (Červená *et al.*, 2019).

Twelve female domestic chickens (1-day old), and 12 male Japanese quails *Coturnix japonica* Temminck & Schlegel, 1849 (2-weeks-old), were housed at the facility and allowed 2 weeks for quarantine and acclimatization. The birds were fed commercial feed mix for chickens and quails during the acclimatization and experiment. The chickens were obtained from a commercial hatchery and were vaccinated against Marek's disease, infectious bursal disease, infectious laryngotracheitis (ILT), avian infectious bronchitis and Newcastle disease. The quails were obtained from a private breeder. Two outbred female Wistar rats (3-months-old), obtained from an accredited breeder, were used as a positive controls.

Experimental infections

L3 larvae of *A. cantonensis* were isolated from experimentally infected *S. octona* snails, that were fed feces of infected rats 35 days prior to the experiment. Larvae were isolated by digestion method (Graeff-Teixeira and Morera, 1995) from the snail body with the shell removed and collected from the sediment. The infective dose was prepared either by manual separation of live larvae using a micropipette with plastic tip (low infection dose), or by diluting (for high infection dose). For the short time period between dose preparation and inoculation, the L3 were stored in physiological saline solution.

Birds of each species were divided into groups – (i) low dose/100 L3: 3 chicken (C1-3) and 3 quails (Q1-3), (ii) high dose/1500 L3: 3 chicken (C4-6) and 3 quails (Q4-6), (iii) snail fed: 3 chicken (C7-9) and 3 quails (Q7-9) and (iv) negative control (C10-12, Q10-12). Inoculation involved inserting an inoculation tube to the crop, while its position was checked by palpation and flushing the prepared infective dose with physiological saline solution. In snail fed groups, live infected *S. octona* snails (7–8 mm in shell height) were carefully inserted using forceps to the back of the oral cavity and the bird was allowed to swallow. Birds were marked by coloured leg rings for identification. All procedures were performed using manual restraint techniques without sedation. Two female laboratory rats (positive controls) were infected the same day with 40 L3 using a gastric tube.

Blood collection, clinical observation, necropsy

Blood from all experimental birds was collected from tarsal vein immediately before inoculation and 7 days after inoculation (dpi). During the post-inoculation period, the health status, food intake and behaviour of birds were observed twice per day. Before the second bloodwork, clinical examination was performed and body condition (Gregory and Robins, 1998) was scored. Haematological analysis was performed by a manual differential count at the clinical laboratory of the Avian and Exotic Animal Clinic of the Faculty of Veterinary Medicine UVPS Brno, while biochemistry values were obtained using the VETSCAN VS2 Chemistry Analyzer. At the end of the experiment (dpi 7) all animals were euthanized using intramuscular administration of ketamine (20 mg kg^{-1}, Narkamon 50 mg mL^{-1} A.U.V. INJ, Bioveta Ivanovice na Hané, Czech Republic) and xylazine (5 mg kg^{-1}, Xylazin 2% A.U.V. INJ, Bioveta Ivanovice na Hané, Czech Republic) as premedication, followed by administration of 1 mL of a mixture of mebezonium iodide, embutramide and tetracaine (T 61 A.U.V. INJ, Intervet International B.V., The Netherlands) intravenously or intracardially. Animals were necropsied, all gross lesions documented and organs were fixed in 4% neutral buffered formaldehyde solution; brain tissue and spinal cord were preserved in 10% neutral buffered formaldehyde. Histological sections were processed standardly and stained by haematoxylin-eosin. Serial sections were made of brain ($n = 8$) and spinal cord ($n = 12$) in each animal.

Results

Clinical observations and necropsy

No experimental birds died during the observation period and mild neurological clinical signs were observed. During the final clinical examination (7 dpi), chicken 2 (low dose) showed bilateral twitching of leg muscles, with normal reflexes; chicken 9 (snail fed) showed delayed righting reflex when put on dorsal recumbency, chicken 5 (high dose) presented with unsteady and uncoordinated gait.

Necropsy revealed a normal body condition score of 2 (on a scale 0–3) in all birds. On gross observation, the fragility of liver tissue was seen in all snail-fed chickens and quails, with focal pale discolourations in two other infected quails. Pale discolouration of kidneys was observed in two low dose chickens. Nematode larvae were not observed, neither during the necropsy nor histologically (see later).

Clinical pathology and histopathology

Five infected quails (all high dose, two snail-fed) showed heteropenia after infection; one high dose quail (Q6) had eosinophilia and two infected chicken (C2 and C6) showed markedly increased eosinophils, 13% and 16%, respectively. Ranges of other slight changes were recorded in both experimental and control groups and are not considered subsequently. Complete results of haematology can be found in Supplement 1 and Supplement 2. Recorded abnormalities in biochemistry profiles are considered irrelevant to *A. cantonensis* infection, as there was no clear pattern of changes affecting only experimental birds. The biochemistry results can be found in Supplement 3 and Supplement 4.

As both heterophils and eosinophils in birds have eosinophilic cytoplasmic granules and are difficult to distinguish in tissues by conventional stains (Barnes and Fletcher, 1996) both are referred to as granulocytes with eosinophilic granules (acidophils).

Areas of lymphoid infiltration in meninges was observed in three infected quails, in one case with the presence of acidophils. Two infected quails showed mild non-specific pneumonia, in one case with the presence of acidophils. Seven infected birds (6 chickens, 1 quail) showed hepatitis, in one case with the presence of acidophils. In high dose chicken 3, the authors saw interstitial focal chronic inflammation in the muscle layer of the stomach with the presence of acidophils. Additionally, chronic interstitial nephritis was observed in two infected chickens (C3 and C6). In several chickens, liver fragility accompanied by vacuolization in hepatocytes was present, which could be attributed to deposition of fat and which is a physiological finding in chickens of this age.

Positive control rats

Control rats checked for L1 shedding by faecal larvoscopy (Beane and Hobbs, 1983) at 45 dpi were positive and showed no clinical signs during the prepatent period.

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Discussion

Several cases of neurostrongyliasis have been documented in birds: wild tawny frogmouths *Podargus strigoides* (Latham, 1801) in southern Queensland (Gelis *et al.*, 2011) and Sydney (Ma *et al.*, 2013) Australia, a captive African pygmy falcon *Polihierax semitorquatus* (Smith, 1836) in California, USA (Burns *et al.*, 2014), gang gang cockatoos *Callocephalon fimbriatum* (Grant, 1803) from an aviary in Sydney, Australia (Reece *et al.*, 2013), and a captive yellow-tailed black cockatoo *Calyptorhynchus funereus* (Shaw, 1794) in Brisbane, Australia (Monks *et al.*, 2005).

Neurological signs such as ataxia, tremors, inability to control movements, paresis and paralysis were described in naturally infected birds (Ma *et al.*, 2013; Reece *et al.*, 2013; Burns *et al.*, 2014). Poor body condition was described in two publications (Gelis *et al.*, 2011; Ma *et al.*, 2013). While three of our experimental chickens showed mild neurological signs consistent with the early stages of nervous larval migration, no severe neurological impairment was observed and body condition scores were normal.

Post-infection haematological and biochemical values in experimental birds do not parallel those of naturally infected birds (Monks *et al.*, 2005; Gelis *et al.*, 2011; Burns *et al.*, 2014). While avian eosinophils are infrequently observed and their function is poorly understood, eosinophilia in birds is still commonly associated with parasitism (Latimer and Bienzle, 2010). Moreover, peripheral eosinophilia and eosinophils in cerebrospinal fluid (CSF) are two diagnostic features of angiostrongyliasis in humans (Wang *et al.*, 2008). We conclude that the changes in blood eosinophils seen in this experiment might be attributed to *A. cantonensis* infection. Post-infection heteropenia was observed in five quails. As all quails, including controls, displayed leukopenia and decline in the number of blood heterophils post-inoculation, this may indicate a process not associated with *A. cantonensis* infection. Also, the changes observed in biochemistry measures post-infection do not follow a clear pattern among infection groups and we do not interpret any of the observed changes as a result of the *A. cantonensis* infection.

In most reported cases of *A. cantonensis* infections in birds, the histopathological findings demonstrated larvae in tissues (Monks *et al.*, 2005; Gelis *et al.*, 2011; Ma *et al.*, 2013; Reece *et al.*, 2013; Burns *et al.*, 2014). On the contrary, no larval nematodes were observed during our study, despite serial sectioning of the CNS, where the larval presence or tissue damage would be expected. Also, previous experimental studies on chicken and various mammals (Alicata, 1963; Wallace and Rosen, 1969) suggest that larvae rarely reach the CNS in chickens, in contrast to rabbits, guinea pigs and mice, which were infected and examined in the same way. Although areas of mild lymphoid infiltration in meninges was found in three infected birds, these findings are probably not caused by experimental infection, as the meningeal signs occur in the later stages of the infection.

In previous studies, some naturally infected birds presented with gastrointestinal signs, with histopathology revealed subacute enteropathy, haemorrhages and oedema in the ventriculus, and granulomas of the muscularis and serosa (Monks *et al.*, 2005; Reece *et al.*, 2013). Larvae have been found occasionally in the liver in tawny frogmouths (Gelis *et al.*, 2011) and numerous small granulomas containing nematodes were observed in the lungs of one infected gang gang cockatoo (Reece *et al.*, 2013). Experimental infection of chickens documented retrieval of larvae from the liver and lungs by use of a Baermann funnel (Wallace and Rosen, 1969). Although some of the observed histopathological changes in our experimental animals might suggest aberrant larval migration through liver, lungs, kidney or GIT, we cannot undeniably attribute them to the infection, as there might be other factors causing them as well.

In our experiment, the *A. cantonensis* larvae were not found in examined tissues, which contrasts with reported natural infections in other avian hosts. We used a maximum of 1500 L3 per experimental bird, which is a dose proven lethal for some mammalian hosts. However, in endemic areas, molluscan intermediate hosts commonly harbour substantially more infective *A. cantonensis* larvae (Li *et al.*, 2008; Tesana *et al.*, 2009; Qvarnstrom *et al.*, 2013; Oliveira *et al.*, 2015; Vitta *et al.*, 2016) and theoretically higher infection doses can elicit more pronounced clinical signs. Feral chickens represent a dominant part of avifauna in many *A. cantonensis* hotspots, including Pacific islands (Gering *et al.*, 2015). The possible role of domestic and feral poultry in the elimination of *A. cantonensis* larvae from human surroundings in hyperendemic areas deserves more attention as a part of prevention of human infections.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182020001869

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Conflicts of interest. None

Ethical standards. The study was authorized by the ethical committee for use of experimental animals at UVPS Brno (approval No. 3-2018 and No. 40-2017) prior to the experiment.

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