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Research paper

Experimental avian philophthalmosis: Evaluation of diagnosis and treatment of chickens infected with *Philophthalmus gralli* (Trematoda: Philophthalmidae)

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ABSTRACT

The trematodes of the genus *Philophthalmus* are eye flukes that cause damage to ocular structures of animals and humans. Despite the increasing number of cases reported in birds, studies related to the diagnosis of subclinical philophthalmosis are lacking, and there are no effective therapeutic regimens available. In the present study, we evaluated the diagnosis and treatment of philophthalmosis in specific pathogen-free chickens (*Gallus gallus domesticus*) experimentally infected with *Philophthalmus gralli*. Four chickens were inoculated with metacercariae of *P. gralli* (20 per eye) obtained from cercariae emerged from naturally infected *Melanoides tuberculata*. From 90 days post-infection, the chickens were subjected to direct ophthalmic examination (DOE) and conjunctival sac lavage (CSL). The latter technique consisted of lavage of each eye with 200 µL sterile saline solution and subsequent microscopical examination of the collected fluid for the presence of eggs of *P. gralli*. The anthelminthic treatment protocols included praziquantel (PZQ) at 10, 50, or 100 mg/kg (single dose given intramuscularly), or fenbendazole (FBZ) at 50 mg/kg (three doses at 24 h-intervals given per os). The treatment protocols were performed at 14 day-intervals between each dosage of PZQ. Chickens developed a minimum of one to more than five adult *P. gralli* per eye, except for one chicken that had a single eye with one parasite. No ocular clinical signs or changes in behavior were noted in any chickens. DOE and CSL were considered techniques with similar sensitivity for the diagnosis of avian philophthalmosis. The data suggested that PZQ and FBZ, at the dosages and schedules employed, are not effective for the complete elimination of *P. gralli*. CSL is proposed as a complementary technique for the diagnosis and monitoring of philophthalmosis post-treatment, especially in subclinical cases. The evaluation of new protocols, routes of administration, and anthelmintic drugs are needed for successful pharmacological treatment of philophthalmosis.

1. Introduction

Species belonging to the genus *Philophthalmus* Looss, 1899 are eye flukes that commonly parasitize birds, but can also be found in mammals, including humans (Nollen and Kanev, 1995). The presence of parasites in the conjunctival sac and nictitating membrane of the infected hosts can cause pathological changes due to irritation and opportunistic infections, resulting in impairment of vision, including blindness, malnutrition, and physical weakness in more severe cases (Mukaratirwa et al., 2005; Rojas et al., 2013; Church et al., 2013). In recent years, a growing number of cases of philophthalmosis have been reported in farm and wild birds in various parts of the world. Most reports are based on post-mortem examination or overt clinical cases with some degree of impairment of vision, such as conjunctivitis, blepharospasm, secondary bacterial infections, blindness and even the loss of the eyeball (Greve and Harrison, 1980; Pinto et al., 2005; Mukaratirwa et al., 2005; Verocai et al., 2009; Meise and Garcia-Parra, 2015; Heneberg et al., 2018). Studies evaluating subclinical infections in live birds are rare (Heneberg et al., 2018), possibly due to the difficulties of diagnosis.

The therapeutic approach commonly undertaken in philophthalmosis is the mechanical removal of individual parasites. Although extremely effective, the procedure demands technical prowess and dexterity for the correct containment of affected animals, including sedation or anesthetic protocols, and use of tools such as micro-spatulas, forceps and tweezers (Mukaratirwa et al., 2005; Church et al., 2013; Literák et al., 2013; Rojas et al., 2013). This approach is difficult for treatment of free-living birds and birds on farms. In this

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sense, anthelmintic treatment appears to be a promising option for the therapy and control of avian philophthalmosis. However, studies evaluating the activity of anthelmintic drugs in birds infected by eye flukes are rare, and the few studies that exist did not suggest effective or safe therapeutic protocols (Nollen and Murray, 1978; Mukaratirwa et al., 2005; Church et al., 2013; Rojas et al., 2013; Heneberg et al., 2018).

Given the difficulties related to the diagnosis of the subclinical philophthalmosis and the fact that pharmacotherapy of the disease remains unstandardized, investigation is needed to solve these problems. In the present study, we evaluated the sensitivity of two parasitological tests: direct ophthalmic examination (DOE) and conjunctival sac lavage (CSL) for the diagnosis of experimental subclinical infection of chickens with *Philophthalmus gralli*. In addition, we evaluated DOE and CSL to determine the efficacy of two widely used anthelmintics, praziquantel (PZQ) and fenbendazole (FBZ).

2. Materials and methods

2.1. Ethics statement

The procedures performed in this study were approved by the Ethics Committee on Animal Use of the Universidade Federal de Minas Gerais (CEUA -UFMG, protocol 20/2016).

2.2. Animals and infection

Thirty-day-old specific pathogen-free (SPF) chickens (*Gallus gallus domesticus*) (n = 4) were used for the experimental infection. The infective forms of *P. gralli* were obtained from naturally infected *Melanoides tuberculata* collected at a water body located in Belo Horizonte, Minas Gerais, Brazil. The metacercariae were thermally excysted with water at 41 °C according to Alicata (1962), counted under a stereomicroscope, and inoculated with the aid of a 25 µL micropipette (Kasvi, Brazil) into the eyes of the birds (n = 20 metacercariae in each eye). After infection, the animals were kept in two shared cages (0.50 m high, 2 m long, 1 m wide), with water and feed *ad libitum*. The animals were individually identified with a mark on the wing feathers made with non-toxic and anti-allergenic paint. Clinical examination of the animals, including the verification of ocular changes due to parasitism, were performed twice a week throughout the experiment.

2.3. Diagnosis

DOE and CSL starting from 90 days post infection (DPI) involved manually restraining the chickens, positioned laterally on the left or right, for the evaluation of each eye. DOE consisted of a careful investigation of the presence of parasites on the ocular structures, including the conjunctival sac, nictitating membrane, cornea, sclera, and eyelids. The parasitic burden of *P. gralli* was estimated semi-quantitatively, according to the following cross scale: (-) absence of visible parasites; (+) presence of one to two parasites; (++) three to five parasites; (+++) more than five parasites or presence of uncountable parasites in cluster. This exam was immediately followed by CSL, consisting of instillation of 200 µl sterile saline solution (0.85% NaCl) with the aid of a plastic Pasteur pipette, followed by immediate re-aspiration of the liquid. In order to increase the contact of the physiological solution with the conjunctival sac, each procedure was performed with three cycles of instillation and re-aspiration. The collected lavage was transferred into microtubes, fixed in 10% formalin and evaluated by optical microscopy (10x objective magnification) for the detection and quantification of *P. gralli* eggs that were classified as viable or non-viable as described by Alicata (1962).

2.4. Anthelminthic treatment

We used the experimental design "before-after" for evaluation of the efficacy of the anthelminthic therapy of chickens experimentally infected with *P. gralli*. DOE and CSL were performed in all animals at 7 and 14 days after each treatment. Given the usually short time required for the elimination of helminths using the evaluated drugs, the presence of *P. gralli* or eggs at 14 days post-treatment was interpreted as therapeutic failure.

Treatment with PZQ (Cestodan® Konig® Laboratories, Santana de Parnaíba, SP, Brazil) at 10, 50, or 100 mg/kg in a single intramuscular (IM) administration was performed at 120, 134, and 148 DPI, respectively. When necessary, the drug solution was divided into two injection sites in the pectoral muscles. At 162 DPI, FBZ (Fencare 4% Premix® Virbac® Laboratories, São Paulo, SP, Brazil) at 50 mg/kg was given in three doses orally at 24 h intervals. After each treatment, the chickens were observed for the occurrence of side-effects. The animal was considered cured if there were negative results for both eyes by DOE and CSL performed after 7 days of treatment.

Three days after all the treatments and examinations were performed (175DPI), all visualized parasites were mechanically removed with the aid of toothless Adson tweezers (Eldo, Brazil). The recovered parasites were counted, pressed between glass slides and fixed in 10% formalin. Subsequently, they were processed according to conventional helminthological techniques, including staining with alum acetic carmine, dehydration in graded series of alcohol, diaphanization in beechwood creosote and mounting on a slide with Canada balsam. Re-examination of the chickens was performed 24 h after mechanical removal of the parasites.

3. Results

3.1. Diagnosis before first treatment

Adult parasites were visualized in the conjunctival sac and under the nictitating membrane (Fig. 1A and B) in 7/8 (88%) of eyes examined by DOE, with 3/4 chickens (75%) having bilateral ocular parasitism. However, the right eye of chicken number 1 was negative in all exams, and the infection of its left eye was mild. The data obtained from semi-quantitative analysis of the parasite burden in each eye of each chicken are shown in Table 1. Eggs of *P. gralli* (viable and non-viable) were recovered by CSL in all seven eyes found positive by DOE (Fig. 1C and D). Moreover, eggs of the parasite were not found by CSL in the eye diagnosed as negative by DOE.

3.2. Treatment

The experimental chickens tolerated the anthelmintic treatments well, and no side-effects were observed, except with PZQ at 100 mg/kg. At this dosage, side-effects such as prostration, hypothermia, and balance deficit were immediately observed after administration in all chickens. All these symptoms subsided within approximately 3 h. In addition, at the site of injection (pectoral muscle), a local rigid nodulation could be detected by palpation, remaining until the end of the experiment.

The anthelmintic treatments did not result in complete elimination of *P. gralli* in all the experimentally infected chickens (Table 1). Treatment with single doses of PZQ at 10 or 50 mg/kg caused no changes in DOE and CSL results (positivity for *P. gralli*), as evaluated at 7 and 14 days post-treatment, while PZQ at 100 mg/kg resulted in negativity for *P. gralli* in both eyes by DOE (but not by CSL) in chicken number 3, as observed 14 days post-treatment. In relation to FBZ at 50 mg/kg (3x q. 24 h), two chickens (1 and 3) were found with both eyes negative by DOE and CSL, performed at 7 days after treatment.

The quantification of eggs obtained by CSL resulted in a mean of 26 (7–66) *P. gralli* eggs, found in 7/8 eyes before treatment. At the end of the evaluation of treatments, 5/8 eyes were found negative for *P. gralli* eggs, and three eyes from two chickens showed one, two, and 55 eggs. A high variability was observed regarding the viability of *P. gralli* eggs