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Research paper

Detection of specific antibodies in cats infected with the lung nematode *Aelurostrongylus abstrusus*

Eva-Maria Zottler[a], Christina Strube[b], Manuela Schnyder[a,*]

[a] University of Zurich, Vetsuisse Faculty, Institute of Parasitology, Winterthurerstrasse 266a, 8057 Zurich, Switzerland
[b] University of Veterinary Medicine Hannover, Institute for Parasitology, Centre for Infection Medicine, Buenteweg 17, 30559 Hannover, Germany

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ABSTRACT

Feline aelurostrongylosis, caused by the metastrongylid nematode *Aelurostrongylus abstrusus*, is an underestimated respiratory parasitosis. Its diagnosis currently mainly relies on the isolation of first stage larvae from fresh faecal samples. The aim of our study was to develop a serological test for the detection of specific antibodies against *A. abstrusus* by ELISA. We used recombinant major sperm protein (MSP) of the bovine lungworm *Dictyocaulus viviparus* as detection antigen and evaluated two different ELISA plates (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) with two different enzyme systems [alkaline phosphatase (AP) and horseradish peroxidase (HRP)]. Sera from cats experimentally (n = 54) and naturally (n = 17) infected with *A. abstrusus* and from randomly selected cats with different medical issues (n = 160) were used to determine sensitivity and specificity. Furthermore, cross-reactions were evaluated using sera from cats naturally (n = 71) and experimentally (n = 8) infected with different nematodes. A sensitivity of 100% was obtained with sera from experimentally infected cats at 10 weeks post infection using MSP on the Immobilizer™ Amino-plate with HRP, while it ranged between 90.5 and 95.2% in the other ELISA set-ups. Using sera from naturally infected cats, a sensitivity of 88.2% (95% confidence interval: 63.6–98.5%) was achieved in all four set-ups. The specificity was 85.2–94.4% in sera from uninfected cats prior to experimental infection and 68.1–90% in randomly selected cats depending on the plate and enzyme system. The number of seropositive cats increased over time post infection. Serological follow-up showed a decrease of antibody levels within 30 days after anthelmintic treatment. Seropositive reactions were observed with sera from stray cats naturally infected with *Toxocara cati, Capillaria* sp., hookworms and Taeniidae; however, coproscopic false negative *A. abstrusus* findings cannot be excluded. The serological detection of specific antibodies against *A. abstrusus* using ELISA requires a single serum sample and therefore represents a valid alternative for reliable individual diagnosis of *A. abstrusus* in cats and facilitates mass screening, overcoming the usually difficult collection of cat faeces.

1. Introduction

The lung nematode *Aelurostrongylus abstrusus* infects cats and other felids and has a worldwide distribution (Hamilton, 1963; Scott, 1973; Szczesna et al., 2006; West et al., 1977). The prevalence of *A. abstrusus* infection ranges from 0.5% (Barutzki and Schaper, 2011) to 57% in symptomatic cats (Jefferies et al., 2010), with variations depending on the tested population and the diagnostic techniques applied. Cats are infected through ingestion of infectious third-stage larvae (L3) from an intermediate (snails and slugs) or paratenic host (mice, reptiles, birds) (Hamilton and McCaw, 1967; Jezewski et al., 2013; Scott, 1973). After migration and further development, the adult worms establish in the lung parenchyma of infected cats, where they reproduce. Females lay eggs, from which the first-stage larvae (L1) hatch. These are coughed up towards the upper respiratory tract, to be swallowed and excreted through the gastrointestinal tract (Hamilton, 1963). The prepatent period ranges from 4 (Losonsky et al., 1983) to 9 weeks post infection (wpi) (Hamilton and McCaw, 1968) with a peak of larval excretion between 60 and 120 days post inoculation (dpi) (Ribeiro and Lima Dos Santos, 2001). Affected cats present with respiratory (Grandi et al., 2005; Hamilton, 1967; Traversa et al., 2008a) and/or unspecific signs (Genchi et al., 2014; Schnyder et al., 2014), or can remain asymptomatic (Genchi et al., 2014; Hamilton, 1963). The severity of clinical signs depends on the number of ingested L3 and of the immune status (Hamilton, 1967; Schnyder et al., 2014). Due to often

* Corresponding author.
E-mail address: manuela.schnyder@uzh.ch (M. Schnyder).

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slowly progressing changes, clinical signs may be subtle and therefore unnoticed by animal owners, even if the pathological changes in the lungs can be considerable (Briggs et al., 2013; Hamilton, 1967). Occasionally, aelurostrongylosis may lead to death (Ellis et al., 2010; Philbey et al., 2014).

A. abstrusus infections induce changes in blood parameters (Grandi et al., 2005; Schnyder et al., 2014; Yildiz et al., 2011) and changes in the lung parenchyma are also visible in radiology (Grandi et al., 2005; Losonsky et al., 1983) and computed tomography (Dennler et al., 2013; Payo-Puente et al., 2005). However, these changes are not pathognomonic. Currently, parasitological diagnosis primarily relies on the detection of L1 in fresh faecal samples using larval migration techniques such as the Baermann-Wetzel method (Deplazes et al., 2016). Alternatively, assays like FLOTAC offer detection and quantification of L1 (Gaglio et al., 2008). Furthermore, diagnosis through the detection of parasite DNA from faecal or pharyngeal swab samples is possible (Traversa et al., 2008b) and the microscopic and cytologic examination of bronchoalveolar lavage (BAL) have been described (Foster et al., 2004). Although these different and partly laborious procedures may be adopted, detecting feline lungworm infections remains challenging, as most of the diagnostic techniques rely on the production and detection of L1, which can be absent (i.e. during prepatency or after reinfections (Hamilton, 1968)) or intermittent, and which need to be differentiated from other larvae present in faeces (Jefferies et al., 2010; Varcasia et al., 2015). In addition, in view of the fact that most of the cats have outdoor access (and are incidentally at higher risk of infection (Beugnet et al., 2014)) and defecate outside, obtaining faecal samples for coproscopic analyses is difficult. Therefore, a serological test for diagnosis of *A. abstrusus* infections represents a desirable alternative.

A recombinant major sperm protein (MSP) has been successfully employed in an enzyme-linked immunosorbent assay (ELISA) for the detection of the cattle lungworm *Dictyocaulus viviparus* (Strube et al., 2009; von Holtum et al., 2008). MSP is a highly conserved protein among nematodes, exclusively expressed by male adult worms (Schnieder, 1993; Ward et al., 1988) and which has also been evaluated for the diagnosis of lungworm infections in seals (Ulrich et al., 2015). The advantages of a recombinant antigen for diagnostic purposes are numerous: the production is standardized and the antigen is of a constant quality (von Holtum et al., 2008), and, importantly, obviates the production of native *A. abstrusus* antigens to be obtained from infected cats or snails.

The aim of this study was to evaluate a new diagnostic approach that detects specific antibodies against *A. abstrusus* in infected cats through serological ELISA using the recombinant bovine lungworm MSP as detection antigen.

2. Material and methods

2.1. Cat sera

a) Well-defined sera were obtained in the frame of previously performed experimental trials (Böhm et al., 2015; Schnyder et al., 2014). Briefly, cats were inoculated with *A. abstrusus* L3 and followed for up to 84 dpi without (n = 22) and with (n = 32) anthelmintic treatment performed between 40–54 dpi with the recommended dose of emodepsid/praziquantel (Profender®, Bayer Animal Health). Infections were confirmed by the presence of L1 in faeces, determined by the modified Baermann-Wetzel method (Deplazes et al., 2016), starting between 35 dpi and 41 dpi, and by necropsy with determination of worm burdens.
b) Sera from overall 17 cats, naturally infected with *A. abstrusus*, were obtained during castration campaigns (n = 11) performed in Switzerland, from patients of the Animal Hospital of the Vetsuisse Faculty of the University of Zurich (n = 4) and from a private veterinary clinic in Switzerland (n = 2). Infections were confirmed by isolation of L1 from faecal samples (Baermann-Wetzel method, Deplazes et al., 2016) and by subsequent PCR performed as previously described (Annoscia et al., 2014).
c) Seventy-one sera from cats naturally infected with different nematodes (*Toxocara cati* n = 35, hookworms n = 5, *Capillaria* sp. n = 16) and cestodes (Taeniidae n = 15) were obtained during castration campaigns (n = 67) or from private patients (n = 4). The diagnosis was based on the result of a combined sedimentation/flotation technique (Deplazes et al., 2016) using saturated zinc chloride solution (specific gravity 1.45). As the amount of faecal material obtained from stray cats (n = 67) was restricted (1–6 g), no larval migration methods were applied with these samples. Samples from the remaining four privately owned cats were instead examined with the Baermann-Wetzel technique: they were all negative for larval detection.
d) In addition, further three sera from cats experimentally infected with *T. cati* and five sera of cats infected with *Ancylostoma tubaeforme* (obtained from experimental infections performed at the Institute for Parasitology at University of Veterinary Medicine Hannover, permitted by the ethics commission of the German Lower Saxony State Office for Consumer Protection and Food Safety under reference number 33.9-42502-05-15A587) were used for the evaluation of cross-reactions.
e) Specificity was determined with 160 randomly selected sera from cats tested at the Clinical Laboratory of the Vetsuisse Faculty of the University of Zurich for various reasons, without suspicion of parasitic infections.

All mentioned studies were performed in compliance with current national laws and regulations after approval by the relative authorities.

2.2. ELISA

The recombinant *D. viviparus* MSP used as detection antigen was produced as previously described (von Holtum et al., 2008). To determine the optimal working combination, two different 96-well plates (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) were tested with two different conjugate enzymes [alkaline phosphatase (AP) and horseradish peroxidase (HRP)]. Optimal antigen-, serum- and conjugate concentrations were determined by titration experiments. All tests included a blank control, a conjugate control as well as positive and negative controls.

2.2.1. Alkaline phosphatase (AP) based enzyme immunoassay

Plates were coated (100 µl/well) with recombinant MSP diluted in 0.1 M carbonate/bicarbonate coating buffer (pH 9.6) at a concentration of 0.125 µg MSP/well and incubated overnight at 4 °C in a humid chamber. The following day plates were washed 4 times with 0.9% NaCl containing 0.3% Tween-20 (NaCl-T) and saturated (300 µl/well) with phosphate buffered saline (pH 7.2) containing 0.2% Tween-20, 0.05% bovine haemoglobin (Sigma-Aldrich, Missouri, United States) and 0.02% NaN_3 (PBS-T) for 30 min at 37 °C. The sera were diluted 1:200 in PBS-T (100 µl/well) and incubated for one h at 37 °C. Following another washing step, the plates were incubated for one h at 37 °C with goat anti-feline IgG alkaline phosphatase conjugate (Southern Biotech, Birmingham, USA) at a dilution of 1:2000 in PBS-T (100 µl/well). After repeating the washing, 100 µl/well of a 1 mg/ml solution of 4-nitrophenyl phosphate (Sigma-Aldrich, Missouri, United States) in 0.05 M carbonate/bicarbonate buffer (pH 9.8) containing 1 mM MgCl were added and incubated at 37 °C. The optical densities were measured