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Research paper

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

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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

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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

after 20 min at 405 nm using a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland).

2.2.2. Horseradish peroxidase (HRP) based enzyme immunoassay

Plates were coated (100 μl/well) with recombinant MSP diluted in 20 mM phosphate-buffered 150 mM saline (PBS-2, pH 7.4) at a concentration of 0.125 μg MSP/well and incubated overnight at 4 °C in a humid chamber. The plates were then washed three times for 5 min with 0.05% Tween in PBS-2 (PBS-2-T) and incubated for one h at 37 °C with sera diluted 1:200 in PBS-2-T (100 μl/well). Following another washing step, the plates were incubated for another h at 37 °C with an HRP-labeled goat anti-feline IgG (Southern Biotech, Birmingham, USA) at a dilution of 1:750 in PBS-2-T (100 μl/well). After the final washing step the wells were filled with 50 μl/well of σ-phenylene-diamine dihydrochloride (Sigma-Aldrich, Missouri, USA) in 25 mM citrate/50 mM phosphate buffer containing 0.04% hydrogen peroxide and incubated in the dark. After 10 min the reaction was stopped by adding 2.5 M sulphuric acid (50 μl/well). The optical densities were measured at 492 nm using a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland).

2.3. Statistical analysis

Microsoft Excel 2010 for Windows (Microsoft Corporation, Redmond, USA) was used to calculate the means and the standard deviations (SD). The exact binomial 95% confidence intervals (CI) were calculated according to Clopper and Pearson (Clopper and Pearson, 1934). Specificity and sensitivity were determined with the following formulas: Sensitivity = number of seropositive animals/number of infected animals of each sample group; Specificity = number of seronegative animals/number of uninfected animals. Receiver operating characteristic (ROC) analysis was used to determine the optimal cut-off values and calculation of corresponding sensitivity and specificity (Microsoft Excel 2010).

2.4. Characterization of A. abstrusus *MSP*

To ensure usability of *D. viviparus* recombinant MSP for detection of anti-*A. abstrusus* MSP antibodies, genomic DNA of *A. abstrusus* L1 was isolated and amplified with degenerate primers as described by Ulrich et al. (2016). Degenerate primer stretches were removed from the sequenced *A. abstrusus* MSP gene before submission to GenBank (accession no. KX618917). For nucleotide and amino acid sequence comparison with *D. viviparus* MSP (accession no. DQ999999), non-coding bases were omitted.

3. Results

3.1. Sensitivity and specificity

The diagnostic value of four different ELISAs, where two different enzymatic conjugate systems (AP and HRP) on two different ELISA plates (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) were combined, were evaluated with sera from cats experimentally or naturally infected with *A. abstrusus* and with randomly selected sera and are shown in Table 1. A single cat among all experimentally inoculated animals remained seronegative throughout the experiment. This cat never shed L1 either and did not harbour adult worms at necropsy; therefore, the animal was excluded from data analysis. Using HRP on the Immobilizer™ Amino-plate, we obtained a sensitivity of 100% testing sera from experimentally infected cats 70 dpi (n = 21), while in the other plate set-ups, sensitivities varied between 90.5 and 95.2%. The assays' sensitivity evaluated with 17 naturally infected cats was of constant 88.2% in all four assay combinations. More in detail, this result is explained by one cat which remained seronegative in all four combinations, while two further cats were alternately negative and positive in two combinations each.

Specificity varied between 85.2 and 94.4% testing uninfected cats prior to experimental infection (n = 54) and between 68.1 and 90% testing random samples from cats tested for different medical reasons (Table 1). Potential cross-reactions of sera obtained from cats with other helminth infections are summarised in Table 2. No cross-reactions were observed with sera from *T. cati* or *A. tubaeforme* experimentally infected cats nor with sera from privately owned animals. Sera from stray cats with other helminth infections were frequently seropositive in all four test combinations: 40% of cats infected with hookworms, between 26.7 and 53.3% of sera from cats with taeniid infections, 31.5–62.5% of sera from cats diagnosed with *Capillaria* sp. and 34.3–51.4% of cats infected with *T. cati* (Table 2).

3.2. Detection of specific antibodies during experimental A. abstrusus *infections*

The seroconversion of cats experimentally infected with *A. abstrusus* and without anthelmintic treatment (n = 21) occurred at the earliest 15 dpi, but more consistently between 28 and 40 dpi (Table 3, Fig. 1). At 70 dpi, all cats were seropositive in the combination of the Amino-plate with the HRP enzyme conjugate system, while the OD values for 1–2 cats were still below the cut-off in the other combinations. The OD values from sera from the five cats followed up to 84 dpi persisted at high levels. No correlation between worm burden detected at necropsy and OD values in ELISAs was observed.

Mean OD values for cats that were treated once (n = 24) or twice (n = 8) with an anthelmintic between 40–54 dpi are also shown in Fig. 1. Treatment was followed by declining OD values in all cats, even for those sera showing ODs below the cut-off before drug administration. Among 15 seropositive cats at 40 dpi using the Maxisorb-plate with AP, only 2 were still positive at 70 dpi. In analogy, 10/18 (Immobilizer™ Amino-plate with AP), 7/18 (Maxisorb-plate with HRP) and 15/21 (Immobilizer™ Amino-plate with HRP) sera were still seropositive 30 days after anthelmintic treatment.

3.3. Characterization of A. abstrusus *MSP*

Sequence alignment of the coding regions of the obtained *A. abstrusus* MSP gene sequence with *D. viviparus* MSP revealed 45 nucleotide substitutions affecting 42 codons (Fig. 2). Forty-four substitutions were synonymous, whereas the substitution 80A > G was non-synonymous, resulting in substitution H27R on the amino acid level. According to the amino acid isofunctionality scale by Gindilis et al. (1998), this substitution is isofunctional.

4. Discussion

To date, the diagnosis of *A. abstrusus* infections in cats is primarily achieved by larval detection in fresh faecal samples. The sensitivity of methods such as the Baermann method or FLOTAC can be increased by multiple faecal examinations. However, due to the low compliance of animal owners for multiple faecal collections and the intrinsic difficulty to obtain faeces from outdoor cats in sufficient amounts and also to guarantee proper storage of the samples, alternative methods are highly desirable (Elsheikha et al., 2016). First attempts to detect antibodies against *A. abstrusus* in cat sera have been made suing immunofluorescence antibody testing (Briggs et al., 2013; Hamilton and Roberts, 1968), where antigen from *A. abstrusus* L1 or L3 were employed. Tests based on native antigens require naturally or experimentally infected animals, while conversely the use of a recombinant antigen offers

Table 1
Evaluation of two different ELISA plates (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) and enzyme systems [horseradish peroxidase (HRP) and alkaline phosphatase (AP)] for the detection of specific antibodies against *Aelurostrongylus abstrusus* in defined cat sera using a recombinant major sperm protein (MSP) of *Dictyocaulus viviparus* as detection antigen. Cut-off values were determined through receiver operating characteristic (ROC). CI: confidence interval.

MSP	Sensitivity% (95% CI)		Specificity% (95% CI)	
	experimentally infected cats[a] (n = 21)	naturally infected cats[a] (n = 17)	negative cats[b] (n = 54)	random samples[c] (n = 160)
Maxisorb plate with HRP	95.2 (76.2–99.9)	88.2 (63.6–98.5)	92.6 (82.1–97.9)	85 (78.5–90.1)
Amino-plate with HRP	100 (86.7–100)	88.2 (63.6–98.5)	92.6 (82.1–97.9)	90 (84.3–94.2)
Maxisorb plate with AP	95.2 (76.2–99.9)	88.2 (63.6–98.5)	85.2 (72.9–93.4)	70.6 (62.9–77.6)
Amino-plate with AP	90.5 (69.6–98.8)	88.2 (63.6–98.5)	94.4 (84.6–98.8)	68.1 (60.3–75.3)

[a] Patent infections (70 days post infection in experimentally infected animals) confirmed by the presence of first-stage larvae of *A. abstrusus* isolated from faeces by the Baermann-Wetzel technique and confirmed by PCR.
[b] Sera from cats prior to experimental infections.
[c] Sera of randomly chosen cats submitted to the Clinical Laboratory of the Vetsuisse Faculty of the University of Zurich by veterinarians for haematological or chemical analyses for different medical reasons.

Table 2
Evaluation of the recombinant major sperm protein (MSP) of *Dictyocaulus viviparus* as antigen in ELISAs for the detection of specific antibodies against *Aelurostrongylus abstrusus* on two different ELISA plate types (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) using horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated anti-feline antibodies, tested with sera of cats with coproscopically detected other helminth infections (naturally infected = nat. inf.; experimentally infected = exp. inf.).

			No. of seropositive cats			
	Toxocara cati, exp.inf. (n = 3)	*Ancylostoma tubaeforme*, exp. inf. (n = 5)	*Toxocara cati*, nat. inf.[a] (n = 35)	*Capillaria* sp., nat. inf.[a] (n = 16)	Hookworms, nat. inf.[a] (n = 5)	Taeniidae, nat. inf.[a] (n = 15)
Maxisorb plate with HRP	0	0	14 (40%)	5 (31.5%)	2 (40%)	4 (26.7%)
Amino-plate with HRP	0	0	16 (45.7%)	8 (50%)	2 (40%)	5 (33.3%)
Maxisorb plate with AP	0	0	12 (34.3%)	7 (43.7%)	2 (40%)	6 (40%)
Amino-plate with AP	0	0	18 (51.4%)	10 (62.5%)	2 (40%)	8 (53.3%)

[a] Potentially tested false negative for *A. abstrusus* based on faecal examination (see Discussion).

Table 3
Number and percentage of seropositive cats tested (n = 21) before and after experimental infection with *Aelurostrongylus abstrusus* (dpi = days post infection). ELISAs were performed with two different plates (Maxisorp and Immobilizer™ Amino-plate, Nunc Roskilde, Denmark) and conjugate enzyme systems. HRP = horseradish peroxidase, AP = alkaline phosphatase.

	Prior to infection (n, %)	15 dpi (n, %)	28 dpi (n, %)	40 dpi (n, %)	70 dpi (n, %)
Maxisorb plate with HRP	0 (0%)	3 (14,3%)	8 (38,1%)	16 (76,2%)	20 (95,2%)
Amino-plate with HRP	3 (14,3%)	4 (19%)	6 (28,6%)	15 (71,4%)	21 (100%)
Maxisorb plate with AP	4 (19%)	1 (4,8%)	3 (14,3%)	13 (61,9%)	20 (95,2%)
Amino-plate with AP	1 (4,8%)	1 (4,8%)	4 (19%)	12 (57,1%)	19 (90,5%)

clear advantages. Through recombinant expression of proteins such as bovine lungworm MSP in bacteria, a well-characterized and inexpensive antigen can be produced in large quantities (von Holtum et al., 2008). Based on its successful usage for the detection of lungworms in cattle (Fiedor et al., 2009; Schunn et al., 2012; Strube et al., 2009; von Holtum et al., 2008) and seals (Ulrich et al., 2015), we evaluated *D. viviparus* MSP in different ELISA plates/conjugate enzyme combinations for the detection of the most important cat lungworm *A. abstrusus*. Contrary to previous studies for detection of the bovine lungworm in cattle sera (Gozdzik et al., 2012; von Holtum et al., 2008), we achieved good discrimination between positive and negative samples also with the Maxisorp-plate. However, the best results for a detection of antibodies against *A. abstrusus* in cats were obtained using the Immobilizer™ Amino-plate and the HRP enzyme system, with high sensitivity and specificity in experimentally and naturally infected cats.

As the MSP represents a highly conserved protein family occurring in nematode sperm only, potential cross-reactions with cestode and trematode infections are not expected (Ulrich et al., 2015). In addition, cattle as well as seal sera from animals with gastrointestinal nematodes did not cross-react (von Holtum et al., 2008; Ulrich et al., 2015). This can be explained by the fact that MSP released from adult male nematodes during copulation gets into the host lumen of the digestive tract, where it is not recognized as an antigen, similar to dietary proteins.

The previously mentioned studies evaluating sero-immunological reactions against *A. abstrusus* in cats were less comprehensive, e.g. when evaluating cross-reactivity. For instance seropositivity of a single cat with roundworm infection obtained by IFAT despite the lack of evidence for an *A. abstrusus* infection could not be explained conclusively (Hamilton and Roberts, 1968). Another IFAT based on native *A. abstrusus* L1 antigen was restricted to few animals and excluded a cross-reaction, as evaluated with a single serum from an *Ancylostoma braziliense* infected animal (Briggs et al., 2013).

Interestingly, in our study all sera from cats with a negative sedimentation/flotation result for *A. abstrusus* but tested positive in our ELISAs were stray cats, in which larval excretion could not be tested with the Baermann method due to a lack of faecal material. In contrast, no seropositivity and therefore no cross-reactions were demonstrated with sera from cats experimentally infected with *T. cati* or *A. tubaeforme* and from privately owned cats infected with *T. cati* and Taeniidae (and likely without *A. abstrusus* infections as determined by the Baermann technique, usually considered as reference method despite its limitations). Although larval migration methods are currently considered the gold standard for detection of lungworm larvae in faeces, a previous study performed in Italy showed that all 40 cats that tested

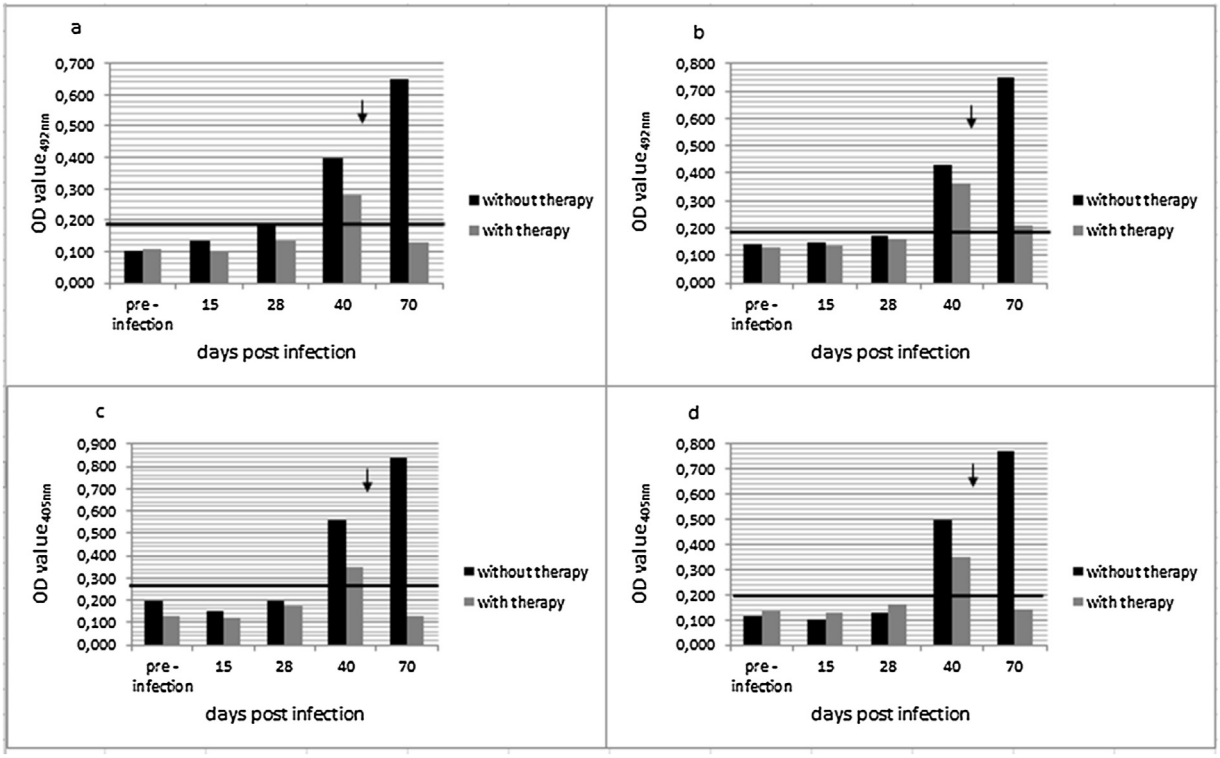

Fig. 1. Specific detection of serum antibodies (IgG) in cats experimentally infected (n = 54) with *Aelurostrongylus abstrusus* in ELISAs using the recombinant major sperm protein (MSP) of *Dictyocaulus viviparus* as antigen on two ELISA plate types (Maxisorp and Immobilizer™ Amino-plate) with horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated anti-feline antibodies. A total of 32 cats were treated with emodepsid/praziquantel spot-on (Profender®) once (n = 24) or twice (n = 8) between 40 and 54 days post infection (dpi), marked with an arrow. The cut-off value, based on ROC-analysis, is represented as a horizontal line. The bars represent the mean optical density (OD) values of the tested sera. (a) Maxisorp plates with HRP; (b) Immobilizer™ Amino-plate with HRP; (c) Maxisorp plates with AP; (d) Immobilizer™ Amino-plate with HRP.

positive for *A. abstrusus* using the Baermann method were also positive when faecal samples were processed by flotation with zinc sulfate solution with a 1.350 specific gravity (Traversa et al., 2008c). Previous studies, including our own ones, based on experimentally infected cats, confirmed that coproscopic false negative results occur during prepatent infections and due to irregularity of larval shedding (Ribeiro and Lima Dos Santos, 2001; Schnyder et al., 2014). Other studies showed that the sensitivity of faecal flotation and combined flotation/sedimentation techniques for the diagnosis of *A. abstrusus* is estimated to be as low as 55.6–66.7% (Lacorcia et al., 2009; Traversa et al., 2008a). This is underpinned by a study from Denmark, where the parasite prevalence in cats may be comparable with that of Switzerland: a comparison between a lung digestion method and the Baermann technique showed a sensitivity of 87% for the detection of *A. abstrusus* infected cats by the Baermann method (Olsen et al., 2015). Importantly, a mean prevalence of 15.6% was observed in that study based on dissection data, which are considered to have the best sensitivity, with regional variations peaking at 72%. The poor performance of coproscopic methods therefore represents a limitation of the study when evaluating cross-reactions, as we can only hypothesize that a substantial number of the serologically positive stray cats may have been infected with *A. abstrusus*, whose presence could not be confirmed coproscopically. First, the limited amount of faecal material did not allow the Baermann analysis and second, the analysis should be performed with faecal samples collected over three consecutive days (as generally recommended, Taubert et al., 2008; www.esccap.org). Both are possible reasons why the parasites may have been missed. Therefore, the prevalence of *A. abstrusus* in cats must be considered generally underestimated, and this is especially true for stray cats, being the most affected population (Beugnet et al., 2014; Traversa et al., 2008c). Consequently, it is tempting to assume that the obtained seropositive results in sera from stray cats from which no *A. abstrusus* L1 could be detected in the combined sedimentation/flotation technique most probably do not represent cross-reactions, but originate from actual *A. abstrusus* infections. This knowledge, in association with the present results, implies that the observed specificity of the ELISA for detection of antibodies against *A. abstrusus* is at least as high as described.

A further limitation of the study is the limited knowledge on cross-reactivity of the ELISAs with other lungworm infections of cats such as *Capillaria aerophila* (syn. *Eucoleus aerophilus*), *Oslerus rostratus* and *Troglostrongylus* sp. Due to high morphological similarity between *Troglostrongylus* sp. and *A. abstrusus* L1, *Troglostrongylus* sp. have been excluded here in naturally infected cats by molecular analyses; their occurrence actually seems to be restricted to southern European countries (Di Cesare et al., 2015; Diakou et al., 2015; Jefferies et al., 2010). *O. rostratus* is a seldom diagnosed parasite (Brianti et al., 2014) and, like *Troglostrongylus* sp., has not been observed in Switzerland so far. Infections with *C. aerophila* may occur, as dogs and in particular foxes are frequently infected with this parasite (Hauser et al., 2015; Magi et al., 2015; Saeed et al., 2006). Sera from cats harbouring these other lungworms of marginal significance (compared to *A. abstrusus*) may also be identified by the same ELISAs since MSP is highly conserved among nematodes. In particular infections with *Troglostrongylus* and *O. rostratus,* both belonging to the superfamily of the Metastrongyloidea like *A. abstrusus,* are likely to be detectable with the ELISA, in contrast to infections with *C. aerophila* (belonging to the Enoplida), which may not be identified in infected animals. These speculations could be of interest in future evaluations and

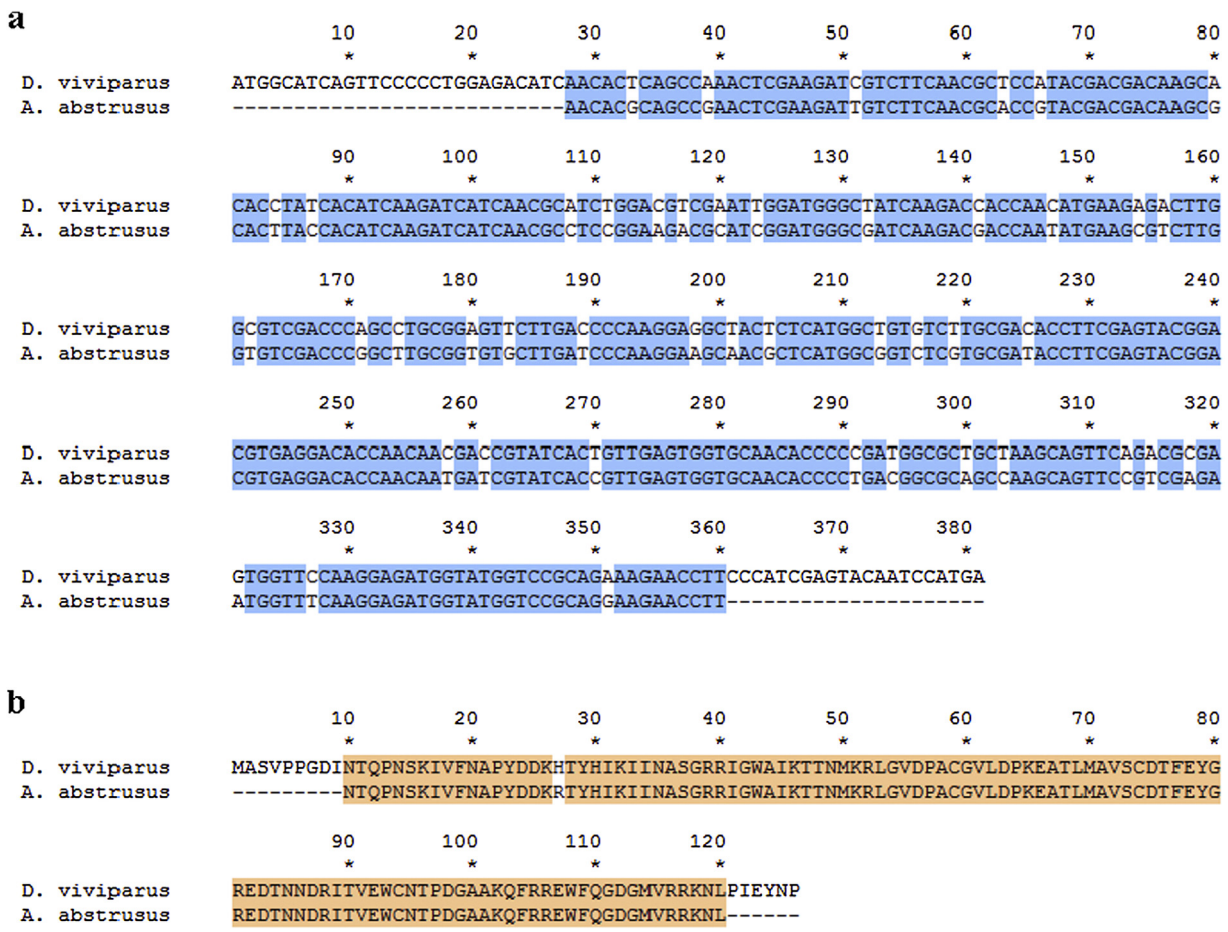

Fig. 2. Coding sequence comparison of *Dictyocaulus viviparus* (accession no. DQ999999) and *Aelurostrongylus abstrusus* MSP (accession no. KX618917). (a) Nucleotide sequence alignment. Missing bases in the *A. abstrusus* sequence represent degenerate primer stretches. (b) Protein sequence alignment. Missing amino acids in the *A. abstrusus* sequence represent degenerate primer stretches.

the results potentially contribute to identifying cats with metastrongylids other than *A. abstrusus*.

In experimentally infected cats without treatment, the proportion of seropositive animals increased over time, reaching 100% after 10 weeks using the Immobilizer™ Amino-plate and the HRP enzyme system (Table 3), and OD values persisted at high levels in the five experimentally inoculated cats that were sampled again around 84 dpi. In chronically infected animals and also in cats that have been re-infected, the clinical picture may not be obvious, despite clear pathological lesions that have been observed e.g. at necropsy or by diagnostic imaging (Grandi et al., 2005; Payo-Puente et al., 2005; Traversa et al., 2008c). Antibodies persisting at high levels over the course of the infection therefore represent a further important advantage compared to faecal examinations in diagnosing *A. abstrusus* infections in chronically infected and also re-infected animals, in which L1 excretion often disappears (Ribeiro and Lima Dos Santos, 2001), precluding correct diagnosis by coproscopic methods. In line with this, experimental trials showed that repeated administration of 50 L3 to young cats induced a high degree of resistance to re-infection, as only one out of 6 cats excreted L1 after being challenged with 800 L3, although few adults and pathological lesions were demonstrated at necropsy (Hamilton, 1969).

Importantly, *A. abstrusus* infections were detected at necropsy in 9% of 54 cats that had died in association with anaesthesia and represented the most frequent cause of pulmonary disease in these animals (Gerdin et al., 2011). Therefore, although *A. abstrusus* infections are not necessarily correlated with clinical disease, serology could be a useful tool to detect unnoticed infections for prevention of anaesthetic-associated deaths. However, the OD values seem not to proportionally reflect the worm burden. Similarly, antibody titres against MSP were not correlated to worm burdens in cattle either: infections with approximately fifty *D. viviparus* L3 induced similar antibody titres as infections with thousand L3 (unpublished results).

Out of the 17 sera collected from naturally infected cats, three (two in each ELISA combination) were seronegative despite larval shedding. One possible explanation might be an individual lack of appropriate immune response to the pathogens when the infection occurs at very young age (one such cat was approximately 2–3 months old, excreting high numbers of L1 (Zottler and Schnyder, 2016)), or due to severe concurrent immunosuppressive viral infections such as the feline leukaemia virus (Buchmann et al., 2010). Seronegative results may also arise in animals in early patency, before worms are mature and induce positive anti-MSP antibody levels, as supported by OD values close to the cut-off (results not shown). Larval shedding starts 4–9 wpi (Hamilton and McCaw, 1968; Schnyder et al., 2014) but seroconversion may be delayed. Based on sera from experimental infections, very few cats were already positive at 15 dpi and all became consistently positive only at a later stage of infection (Table 3): highest (100%) sensitivity was reached at 70 dpi. As the MSP is exclusively expressed by adult male worms (Schnieder, 1992; von Holtum et al., 2008), positive results before patency are not expected. The same, i.e. negative ELISA results after 4–9 wpi in infected cats, may apply in the unlikely case of infection with female parasites only.

5. Conclusion

As pathological changes in the lungs (not necessarily accompanied by evident clinical signs) of cats infected with *A. abstrusus* may grow in importance over time (Hamilton, 1967; Schnyder et al., 2014), diagnostic tools with improved sensitivity are needed. The identification of infected cats performed through serological detection of specific antibodies by ELISA has substantial advantages compared to coproscopic analyses, which are ideally performed on multiple freshly collected samples. Considering that cats with outdoor access primarily defecate outside, faecal sample collection can be highly challenging. For serology, instead, a single serum sample is required and test results are delivered within hours. Also, infected cats can be identified even if L1 are absent from faeces, as observed during late prepatency, chronic infections or with reinfections. The test can additionally be used for follow-up measurements after anthelmintic treatment, although it is advisable to repeat serology earliest 6 weeks after anthelmintic treatment. If then the results are still positive, a re-infection is not to be excluded; in that situation, it is recommended to repeat the test again after a couple of weeks.

ELISAs are very useful tools for individual diagnosis of cats and are also suitable for mass-screening. The presented ELISA relies on a recombinant antigen, obviating the need for native antigens preparation, which require parasite maintenance in live animals. Based on negative serology we were able to confirm an unsuccessful experimental inoculation in a single cat. The test can be therefore implemented in experimental trials, with the aim to avoid unnecessary necropsy for parasite isolation. Furthermore, although the detection of antibodies may indicate exposure to the parasite only, the assay could be used for population studies and improve the epidemiological knowledge of this potentially underdiagnosed feline lung parasite.

Conflict of interest

The authors have no conflict of interest to declare.

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