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Short communication

Seroprevalence of antibodies against the cat lungworm *Aelurostrongylus abstrusus* in cats from endemic areas of Italy

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ABSTRACT

Aelurostrongylus abstrusus (Nematoda, Metastrongyloidea) is a worldwide occurring lungworm causing verminous pneumonia in cats. To date the Baermann method is the most used procedure to diagnose *A. abstrusus* infection by isolating first stage larvae from faeces, though its sensitivity and specificity can be impaired by several factors. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against *A. abstrusus* has been recently developed as a diagnostic alternative. The present study evaluated the seroprevalence for *A. abstrusus* infection in cats from two endemic areas of Italy. Overall, 250 sera were sampled and tested for the presence of antibodies against *A. abstrusus*. Based on the results obtained from 20 cats proven to be infected by *A. abstrusus* using Baermann technique and molecular methods, and from 20 negative cats (Subset A), a cut off value of 0.347 optical density (OD) was determined, leading to a sensitivity of 95% and a specificity of 100%. Two-hundred and ten cats (142 and 68 from Abruzzo and Umbria regions, respectively) were included in Subset B (i.e. 202 negative by Baermann examination and 8 positive for *Troglostrongylus brevior*). Antibodies against *A. abstrusus* were detected in forty-five (21.4%, 95% CI: 16.1–27.6%) samples. This study confirms the occurrence of *A. abstrusus* in endemic areas of Italy and indicates that one-fifth of randomly selected cats have or had a lungworm infection with production of antibodies.

1. Introduction

Aelurostrongylus abstrusus (Metastrongyloidea, Angiostrongylidae) is the most important respiratory nematode of domestic cats in Europe and other continents (Di Cesare et al., 2015a; Giannelli et al., 2017). This nematode (the "cat lungworm") lives in the bronchioles and alveolar ducts of the definitive hosts, which become infected by ingesting intermediate (i.e. slugs and snails) or paratenic hosts (i.e. preyed rodents, birds or reptiles) (Jeżewski et al., 2013; Deplazes et al., 2016).

Feline aelurostrongylosis presents with various clinical manifestations, ranging from subclinical and subtle signs to interstitial bronchopneumonia with cough, sneezing, dyspnoea and abdominal breathing, possibly leading to death especially in young and/or immunocompromised individuals (Crisi et al., 2017; Genchi et al., 2014). Importantly, cats also often present with unspecific or absent clinical signs (Crisi et al., 2017; Genchi et al., 2014; Schnyder et al., 2014). In the past few years there has been an increasing awareness about the impact of this parasite on cat health, on its epidemiology and on treatment and prevention of the infection (Conboy, 2009).

Clinical and epidemiological studies on *A. abstrusus* mainly rely on the detection of first stage larvae (L1s), shed by cats with their faeces, *via* larval migration with the Baermann method that is considered the *standard* technique to diagnose *A. abstrusus* infections (Traversa and Di Cesare, 2016). Nonetheless, the sensitivity and specificity of the Baermann method can be impaired by different intrinsic and extrinsic factors. False negative results occur due to prepatency, and/or intermittent larval shedding due to scarce parasite load or during chronic infections (Ribeiro and Lima, 2001). Repeated examinations are recommended to increase its sensitivity (Schnyder et al., 2014). The L1s identification is operator-dependent because morphological and morphometric differentiation of *A. abstrusus* from other nematode stages that can be present in cat faeces is necessary (Brianti et al., 2012). L1s of *A. abstrusus* are

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recognized by their length, commonly considered to be 360–400 µm and by the tail and head features, despite shorter or longer larvae have been documented (Traversa and Di Cesare, 2013; Giannelli et al., 2017).

Genetic approaches are able to detect *A. abstrusus* DNA in pharyngeal mucous or faeces of infected cats, allowing the simultaneous discrimination from other extra-intestinal feline nematodes (Traversa et al., 2008; Di Cesare et al., 2015b). Nonetheless, in some cases (e.g. lack of owner compliance in collecting samples, cats with outdoor access, unavailability of purposed equipment), copromicroscopic and molecular assays cannot be applied. Therefore, alternative diagnostic techniques are desirable. The first attempt of a serological test for detecting *A. abstrusus* antibodies was an indirect fluorescent antibody test (IFAT) that was impaired by cross-reactivity with other cat parasites (Hamilton and Roberts, 1968). Later, a similar assay showed to be promising in detecting antibodies in natural and induced infections and future work was awaited towards more complete data on its usefulness, especially by investigating its specificity (Briggs et al., 2013). No commercial serological test is available.

A significant progress has been recently made with an enzyme-linked immunosorbent assay (ELISA) using recombinant major sperm protein (MSP) derived from the bovine lungworm *Dictyocaulus viviparus* initially developed for the detection of antibodies against lungworms in cattle (von Holtum et al., 2008) and successfully adapted for lungworm antibody detection in seals (Ulrich et al., 2015), and then evaluated for the detection of antibodies against *A. abstrusus* in cats (Zottler et al., 2017). Based on the results achieved in the test validation and in a study evaluating the seroprevalence of *A. abstrusus* in cats from Switzerland, this assay has been considered particularly useful for both large-scale screening in sero-epidemiological surveys and for confirmation of clinical cases of *A. abstrusus* infections (Zottler et al., 2017; Gueldner et al., 2019). This study investigated its usefulness using sera samples collected from cats with a known parasitological status and coming from geographic areas that are considered highly endemic for the most important respiratory parasites of cats.

2. Materials and methods

2.1. Cat sera

Individual serum samples of 250 cats from two different geographic areas of Italy were collected, i.e. 162 from Abruzzo (Site A) and 88 from Umbria (Site B) region, both endemic for major felid respiratory nematodes (Di Cesare et al., 2015a). All cats were enrolled in the study only after the owner's consent, and sera samples were obtained from surplus material of blood sampling performed for other medical checks. The samples were divided in two subsets, i.e. sera of cats whose parasitological status was previously determined using copromicroscopic (i.e. Baermann examination and floatation) and molecular tests (Subset A), or only with copromicroscopic examination (Subset B).

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 (1934).

2.1.1. Subset A

Twenty sera (16 and 4 from Site A and B, respectively) from cats infected with *A. abstrusus* and 20 sera (12 from Site A and 8 from site B, respectively) from cats copromicroscopically negative for lungworms were used. Of the positive cats, two were infected also with *Troglostrongylus brevior*. The parasitological status was determined by microscopic identification of L1s isolated by the Baermann method (Traversa and Di Cesare, 2013; Giannelli et al., 2017), followed by a molecular confirmation (Di Cesare et al., 2015b). Receiver operating characteristic (ROC) analysis (Zweig and Campbell, 1993) was applied to determine the optimal cut-off value.

2.1.2. Subset B

Two-hundred and ten cats (142 and 68 from Site A and B respectively) were included, i.e. 202 negative for lungworms by Baermann examination and 8 (six and two from Site A and B respectively) positive for *T. brevior*, identified based on morphological and morphometric keys (Brianti et al., 2012). Among them, 31 cats were owned and 179 cats were stray or living in colonies.

2.2. ELISA

Sera were tested for the presence of *A. abstrusus* antibodies as previously described (Zottler et al., 2017), with modifications. In detail, Immobilizer™ Amino-plates (Nunc Roskilde, Denmark) were coated (100 µl/well) with recombinant major sperm protein (MSP) of *D. viviparus* diluted in 20 mM phosphate-buffered saline (PBS) (150 mM pH 7.4) at a concentration of 0.250 µg MSP/well. The plates were incubated overnight at 4 °C in a humid chamber, washed three times for 5 min with PBS-Tween 0.05% and incubated for one hour at 37 °C with sera diluted 1:200 in PBS-Tween 0.05% (100 µl/well). After further washing steps, the plates were incubated for one hour at 37 °C with HRP-labeled goat anti-feline IgG (Southern Biotech, Birmingham, USA) at a dilution of 1:9000 in PBS-Tween 0.05% (100 µl/well). Final washing steps were performed and 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 (OD) were initially measured at 450 nm to determine the exact moment for stopping the reaction with 2.5 M sulphuric acid (50ul/well), subsequently the plate was read at 492 nm. using a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland). A reference serum was added twice on each plate to calculate a correction factor for adjustment between plates (Schnyder et al., 2011).

3. Results

Based on copromicroscopic, molecular and serological results of samples from Subset A and ROC analysis, a cut off of 0.347 OD was determined as optimal for the ELISA. Forty-five out of 210 samples from Subset B (21.4%; CI: 16.1–27.6%) had OD values above the cut off and were therefore considered seropositive for detection of *A. abstrusus* antibodies: 28/142 (19.7%; CI: 13.5–27.2%) and 17/68 (25%; CI: 15.3–37.0%) from Sites A and B, respectively, suggesting no significant difference between the prevalence of the two sites. Out of 31 owned cats from Subset B, 4 (12.9%; CI: 3.6–29.8%) resulted seropositive, while 41 (22.9%; CI: 17.0–29.8) of the 179 stray cats or living in colonies were seropositive. Two cats of Subset A positive for both *A. abstrusus* and *T. brevior* by larval excretion and confirmed by PCR were seropositive. Of the eight cats of Subset B positive for L1s of *T. brevior* by Baermann examination (but not confirmed by PCR), three were seropositive and 5 seronegative.

4. Discussion

This is the first serological field study relying on a recently developed ELISA for the detection of antibodies *vs* the cat lungworm *A. abstrusus* performed in cats in Italy and, more importantly, in study areas that are at high risk for felid lungworms (Di Cesare et al., 2015a). The values here obtained can evidently not be directly compared with previous studies carried out in the same areas using the Baermann method. Indeed, these previous studies demonstrated lower rates of infection for *A. abstrusus*, ranging from 3.3% to 18.3% (Traversa et al., 2008; Di Cesare et al., 2015a). Copromicroscopic results from more southern mainland Italy, Sardinia and Sicily showed a prevalence of 11.6–16.7%, while the prevalence in further 9 European countries varied between 0.8 and 35.8%, and no cat positive for lungworms were

detected from Austria and United Kingdom (Giannelli et al., 2017). The data obtained in a recent extensive survey performed in cats from Switzerland demonstrate an overall seroprevalence of 10.7% for *A. abstrusus* antibodies (Gueldner et al., 2019). Switzerland was not considered at high risk for *A. abstrusus* infections as previous studies carried out by copromicroscopy have shown a rate of infection of 0.8–2.3% (Giannelli et al., 2017; Zottler et al., 2019). The here presented data confirm that antibody detection by ELISA can be adopted for mass-screening, identifying a higher number of *A. abstrusus* exposed cats from both high and low risk areas. Contradicting results between serology and Baermann examination, in particular seropositive cats with absence of lungworm larvae in faeces, may indicate a parasite exposure rather than a current infection, as antibodies can persist for weeks after anthelmintic treatment (Zottler et al., 2017). Additionally, in experimental infections antibodies were detected as early as 2 weeks (i.e. before patency), contributing to explain antibody positive but larval negative results. However, and more importantly, larval excretion is known to stop in chronically and/or repeatedly infected cats (Ribeiro and Lima, 2001; Schnyder et al., 2014), while antibodies persist at high levels (Zottler et al., 2017). At last, single false positive results due to potential cross-reactions need to be considered. The MSP used as recombinant detection antigen is a nematode—specific protein, precluding from cross-reactions against trematodes and cestodes. Furthermore, the previously performed validation study of the here employed ELISA (Zottler et al., 2017) showed absence of cross-reactions in sera from cats experimentally infected with major cat nematodes, i.e. roundworms and hookworms. However, a limitation of the serological diagnosis of *A. abstrusus* infection by antibody detection is given by poor knowledge on possible cross-reactivity of the ELISA with other lungworm infections, in particular with the metastrongyloids *Troglostrongylus* spp. and *Oslerus rostratus*. In this study, samples positive for both *A. abstrusus* and *T. brevior* by larval excretion and confirmed by PCR were confirmed seropositive. Interestingly, samples only positive for L1s of *T. brevior* (but not confirmed by PCR) were partially seronegative and partially seropositive. It therefore remains undetermined if the three seropositive cats scored positive due to cross-reaction or for other reasons, i.e. concurrent *A. abstrusus* infection with no larval shedding or a previous exposure with persisting antibodies. Therefore, further studies evaluating *T. brevior* positive cats by this ELISA are warranted. Potential cross-reactions with *O. rostratus* are instead of less concern considering that this parasite is extremely rare in domestic cat populations, even in areas where *A. abstrusus* and *T. brevior* occur with high infection rates (Di Cesare et al., 2015a; Giannelli et al., 2017). Finally, cross-reactions against the trichurid and therefore taxonomically not closely related respiratory nematode *Capillaria aerophila* are unlikely (Zottler et al., 2017).

The cats investigated in the present study were selected randomly and therefore may allow deductions for a larger cat population. In the two selected study areas the endemic presence of lungworms was not only confirmed, but the results indicate that one fifth of the cats had a lungworm infection at the moment of sampling or had persisting antibodies. The higher positivity rate in stray cats or animals living in colonies (but with large roaming areas) is not surprising considering the biological life cycle of *A. abstrusus* and the frequent availability of preys acting as paratenic hosts for free-roaming cats, compared to owned pets which usually have no need to nourish on paratenic hosts.

Innovative diagnostic assays (e.g. in-clinic tests able to detect antibodies or antigens circulating in the bloodstream, or coproantigens in the faeces) will contribute to increase knowledge and disease awareness, as observed in the last decade, in analogy, for canine angiostrongylosis (Schnyder et al., 2017). Such assays may be used especially for individual clinical suspect cases. This is of importance when laboratory examinations are impaired by low compliance of cat owners, i.e. for repeated stool collections due to the intrinsic difficulties in collecting faeces from cats living outdoor. Another practical implication of the here adopted serological tool is its ability to detect subclinical infections, i.e. before interventions that require anaesthesia: identifying infected animals before the interventions may contribute preventing anaesthetic-associated deaths that can occur in cats with unnoticed lungworm infections (Gerdin et al., 2011). Furthermore, this assay can be used in a clinical follow-up for evaluating, along with other parameters (e.g. clinical signs, diagnostic imaging), the efficacy of anthelmintics in treating or preventing *A. abstrusus* infections, towards innovative methods able to circumvent the need of experimental infections and/or necropsies of laboratory animals (Traversa and Joachim, 2018).

5. Conclusion

As infected cats may not necessarily present with respiratory distress but often present with unspecific or absent clinical signs, it is important that lungworms obtain attention from the scientific community and veterinarians. The present results support the hypothesis that field surveys based on antibody detection allow to overcome difficulties related to copromicroscopic examinations and identify a higher rate of infected and/or exposed animals.

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