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Case Report

First molecular identification of *Aelurostrongylus abstrusus* in a cat presenting severe respiratory disease from Israel

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ABSTRACT

Feline lung worm infection is increasingly reported in recent years, and recognized as a cause for respiratory disease in cats. *Aelurostrongylus abstrusus* is regarded as the most prevalent cause of such cases. Infective L3 larvae carried in gastropods and paratenic hosts infect felines, developing to adult worms that reside in the lungs' parenchyma and may cause verminous pneumonia. The L1 larvae hatch from eggs deposited in the lung, and are released to the environment by either feces or sputum. While the majority of epidemiological information regarding *A. abstrusus* originates in European countries, recent studies have shown that it is also found around the Mediterranean basin, as far east as Turkey and Cyprus. A local domestic cat from Israel showing signs of respiratory illness was diagnosed with aelurostrongylosis, confirmed by both morphological and molecular tools. Presence in Israel of this nematode was previously reported in 1949, with no further mentions since. ITS-2 sequence of the isolated larvae was highly similar to that of *A. abstrusus* from domestic cats from Italy. These findings show that distribution of *A. abstrusus* stretch to the eastern shores of the Mediterranean, and that this nematode should be considered as a cause for respiratory disease in cats in Israel and the surrounding countries.

1. Introduction

Parasitic helminth infection of the respiratory tract is an emerging disease of cats, affecting animals of all ages (Pennisi et al., 2015; Giannelli et al., 2017). The causative agent for this condition is in most cases *Aelurostrongylus abstrusus,* but also *Capillaria aerophila, Troglostrongylus brevior, Paragonimus* sp. and others (Pennisi et al., 2015; Traversa and Di Cesare, 2016). *A. abstrusus* (Metastrongyloidea, Angiostrongylidae) is a parasitic gastropod-borne nematode found mainly in domestic cats (*Felis catus*) (Traversa and Di Cesare, 2013). Aelurostrongylosis may be present as a sub-clinical infection, but may also cause respiratory illness varying from mild to severe or even fatal if left untreated (Elsheikha et al., 2016; Traversa and Di Cesare, 2016). It has a wide geographic distribution, with reports from Europe, South America, and Australia (Elsheikha et al., 2016; Giannelli et al., 2017). In the Mediterranean Basin area, the presence of *A. abstrusus* has been reported in recent years from Portugal, through Italy and Greece, and as far east as Turkey and Cyprus (Tüzer et al., 2002; Giannelli et al., 2017; Diakou et al., 2017). A report published by Gerichter in 1949 included a detailed morphological description of adults and larvae of *A. abstrusus* found in cats from Israel, with mention of previous findings of this worm by other researchers (Gerichter, 1949). However, no further reports have been made of any lungworm infection in felines from Israel since this detailed account. The present report describes lungworm infection in a domestic cat from northern Israel presenting severe respiratory disease, including the detection of *A. abstrusus* larvae by morphological and molecular methods.

2. Materials and methods

2.1. Animal

A 4 months old male cat was presented to a veterinary clinic in the Lower Galilee region of northern Israel, with a chief complaint of labored breathing for several days. The cat was privately owned, intact, living indoors with outdoor access to a rural environment. It was

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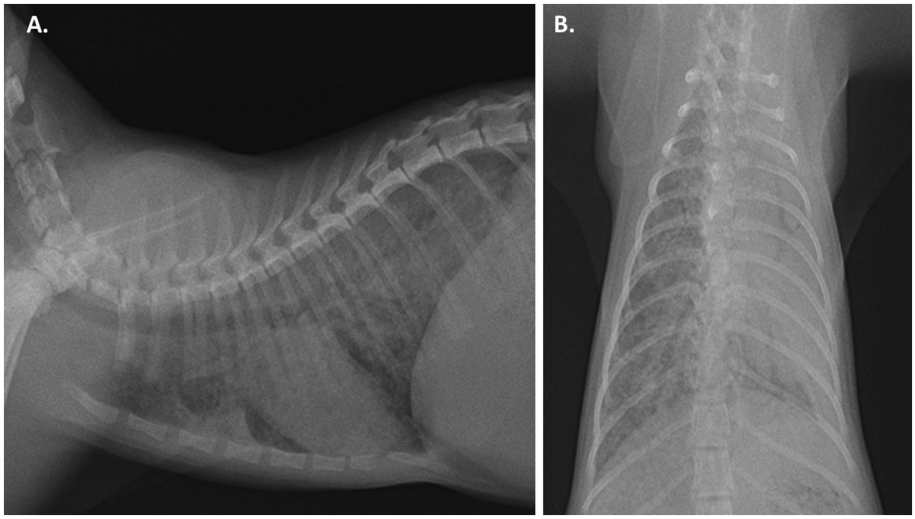

Fig. 1. Thoracic radiograph of the young cat diagnosed with aleurostrongylosis. A diffused and multifocal alveolar pattern is present across most of the lungs field, as well as a nodular interstitial component. A. right lateral view. B. ventrodorsal view.

adopted 3 months earlier, and had no known travel history outside Israel. Physical examination revealed cachexia, increased expiratory effort and elevated body temperature of 39.7 °C (normal range 37–38 °c). Complete blood count (CBC) demonstrated high white blood cells count (46.3×10^6/μL, normal range $5.5–19.5 \times 10^6$/μl), and neutrophilia with no eosinophilia was noted in blood smear evaluation. Thoracic radiographs showed a diffuse and multifocal alveolar pattern across most of the lungs field, with a nodular interstitial component (Fig. 1).

2.2. Procedures

The cat was initially treated with amoxicillin clavulanate (Amoxy/Clav 150 mg/mL, A. VetSupply, Rishon Lezion, Israel), at 20 mg/kg twice per day, with no observed improvement following one week of treatment. Therefore, marbofloxacin (Marbocyl veterinary 20 mg tabs, Linevitz Eliezer, Even Yehuda, Israel) at 10 mg/kg once per day and prednisone (Prednisone 5 mg tabs, Rekah Pharmaceutical Products LTD, Holon, Israel) 2 mg/kg twice per day were added to the treatment. The prednisone dosage was halved after 2 weeks. A second CBC was done after one month and at this time, the white blood cells count was within the normal range (16.25×10^6/μl). However, the labored breathing persisted, and findings in thoracic radiographs were similar to previous ones. Thus, 2.5 months after the first checkup the cat underwent bronchoscopy followed by a bronchoalveolar lavage (BAL). The bronchoscopy demonstrated no visible pathology or interference to airflow in the trachea up to the level of the bronchial bifurcation, and BAL fluid sample was submitted for cytological evaluation. The BAL fluid contained a high number of coiled first-stage (L1) helminth larvae, often found in large cohesive bundles. In addition, intact and degenerate neutrophils were abundant, as well as scattered intact eosinophils. The initial diagnosis was of severe parasitic infection, suggestive of *A. abstrusus*, with concomitant suppurative inflammation. The BAL sample was sent for further morphologic and molecular characterization at the Kimron Veterinary Institute, Ministry of Agriculture, and the Koret School of Veterinary Medicine, the Hebrew University of Jerusalem. Following the initial diagnosis, the cat was administered fenbendazole (Fenbendazole, 100 mg/mL, Vetmarket, Shoham, Israel) at 50 mg/kg once daily for 3 weeks, together with amoxicillin clavulanate at 20 mg/kg twice per day, and prednisone was gradually tapered. The cat's breathing markedly improved following this treatment, until a full recovery was achieved. A fecal sample collected after completion of the treatment tested negative for helminth larvae and eggs using flotation in zinc sulphate and the Baermann technique.

2.3. Morphologic identification

For larvae identification, 20 μL of the BAL fluid were examined under a light microscope (Zeiss Primo Star, ZEISS, Germany) equipped with a digital camera (AxioCam ERc 5 s, ZEISS, Germany) at 100× and 400× magnifications. The total body length and width of 10 larva were calculated using the ImageJ software (Schneider et al., 2012). Digital line drawing of the posterior end of the larvae was produced using the InkScape 0.91 software (Free Software Foundation Inc., Boston, MA, USA). The remaining BAL fluid was stored at −80 °C for further analyses.

2.4. DNA extraction and PCR amplification

One milliliter of the BAL fluid was concentrated by centrifugation at 4000 *xg* for 4 min and the supernatant was discarded. DNA was extracted from the pellet using the Qiagen DNeasy Tissue & Blood kit (Qiagen, Hilden, Germany) and eluted in 50 μL of ATE buffer.

A 330 bp fragment of the internal transcribed spacer 2 (ITS-2) of *A. abstrusus* was amplified using primers AabFor (5'-GTAACAACGATATT GGTACTATG-3′) (Traversa et al., 2008) and NC2 (5'-TTAGTTTCTTTT CCTCCGCT-3′) (Gasser et al., 1993). The reactions included primers at a final concentration of 100 nM and 1 μL of DNA per 25 μL total reaction volume in PCR ready-to-use tubes (Syntezza Bioscience Ltd., Israel). The PCR thermal profile consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min, and a final elongation at 72 °C for 5 min. DNA of *A. abstrusus, Troglostrongylus brevior* and *Ancylostoma caninum* were used as positive controls of the reaction and ultra-pure water (Biological Industries, Kibbutz Beit-Haemek, Israel) as a non-template control (NTC). After this, the PCR products were cloned into pCR 2-TOPO vectors using the Invitrogen TOPO TA cloning kit (Life Technologies, ThermoFisher Scientific Inc., USA) with white-blue colony screening protocol, according to the manufacturer's instructions. Then, plasmids were extracted from three different colonies using the FB Plasmid Miniprep kit (FairBiotech Corp., Republic of China) and the cloned fragments were amplified by conventional PCR using universal primers M13-F and M13-R. The primers were employed at a final concentration of 200 mM and 2 μL of DNA were added per 25 μL total reaction volume in PCR ready-to-use tubes (Syntezza Bioscience Ltd., Israel). Finally, the amplicons were purified (Exo-SAP, New England Bio-Labs Inc., USA) and

sequenced using the Big Dye Terminator cycle sequencing chemistry from Applied Biosystems ABI3700 DNA Analyzer and the ABI's Data Collection and Sequence Analysis software (Applied Biosystems, ThermoFisher Scientific Inc., USA).

2.5. Phylogenetic analysis

The ITS-2 sequence obtained herein was aligned with other *A. abstrusus* sequences available in GenBank with the ClustalW algorithm using the MEGA 7.0 software (Kumar et al., 2016). In addition, *Metastrongylus* spp. and *Angiostrongylus vasorum* ITS-2 sequences were used as outgroups for the phylogenetic analysis. The best nucleotide substitution model was chosen according to the AIC as determined by MEGA 7.0. After this, a Bayesian inference phylogenetic tree was generated using 10^7 MCMC chains and a log of every 10^3 trees in the BEAST package 1.8.4 (Drummond and Rambaut, 2007). The convergence of the chains was evaluated using the Tracer 1.6.0 software, the generated trees were summarized in the TreeAnotator 1.8.4 and visualized in the FigTree 1.4.3 (Drummond and Rambaut, 2007).

3. Results

All 10 larvae recovered from the BAL fluid and examined presented a single, uniform morphology, compatible with previous descriptions of *A. abstrusus* L1 larvae (Gerichter, 1949; Giannelli et al., 2017) (Fig. 2a). Briefly, the larvae were 389 ± 0.055 µm long and 20 ± 2 µm wide. The anterior end was blunt, with a short vestibule that led to a rhabditoid esophagus. The tip of the posterior end was S-shaped with a spine in its dorsal side (Fig. 2b).

The obtained ITS-2 sequence was 99% identical to *A. abstrusus* (DQ372965.2) from a cat in the region of Abruzzo, Italy. It was deposited at GenBank under the accession number MH593881. The phylogenetic tree showed that the *A. abstrusus* obtained from felids of different countries were closely related. However, low posterior probabilities were obtained for the inner branches within this clade (Fig. 3).

4. Discussion

Infection with lungworms are increasingly reported in cats in recent years and are considered to cause an emerging clinical disease (Traversa et al., 2015; Penagos-Tabares et al., 2018). *Aelurostrongylus abstrusus* may be present in cats as a sub-clinical infection (Elsheikha et al., 2016; Giannelli et al., 2017), however some patients present with clinical respiratory disease signs and severe dyspnea (Elsheikha et al., 2016; Deplazes et al., 2016). The finding of *A. abstrusus* in Israel, 69 years after the previous report, raises the question whether this nematode was circulating in the country unnoticed, or was re-introduced recently after being absent for decades. None of the gastropods species previously reported as intermediate host for this nematode are indigenous for Israel (Roll et al., 2009; Elsheikha et al., 2016; Cardillo et al., 2018). However, several non-indigenous land snail species such as *Helix aspersa* and *Rumina decullata* are invading species in Israel, and are presumed to have stable populations in parts of the country (Roll et al., 2009). In addition, *Helix engaddensis* as well as other Helicidae species are endemic and have a wide distribution in Israel (Heller, 2009), their capacity as intermediate hosts of *A. abstrusus* needs to be verified. The mild Mediterranean climate of central and northern Israel favors the buildup of large populations of feral cats, as well as outdoor activity of domestic cats, thus readily sustaining possible lungworm transmission. Since *T. brevior* was described for the first time as a species in the very same report by Gerichter in wild cats from Israel (Gerichter, 1949), it may also be present in the cat population of Israel. In the case presented here, the inflicted cat suffered from severe clinical signs including anorexia, fever and dyspnea with radiographic evidence for bronchopneumonia. The fact that a full recovery was achieved only after anthelmintic treatment, suggests that aelurostrongylosis was the primary etiology. Coprological testing was not done initially in this case, even though it is cheap, simple to perform and non-invasive technique for lungworm larvae detection. This resulted from the fact that lungworm infection was not considered in the differential diagnosis, as in other reports (Soares et al., 2017; Penagos-Tabares et al., 2018). Hence, there is a need to publish such cases and to increase the awareness of aelurostrongylosis in cats demonstrating respiratory signs in areas where this is not known as a common clinical problem. In conclusion, this report of *A. abstrusus* in Israel follows 69 years after the previous report of the species in the country. It raises the question whether this is an endemic or a re-emerging pathogen, and points to *A. abstrusus* as a cause of respiratory disease in felines in this area. Therefore, a survey of healthy and clinically ill cats is needed to estimate the prevalence and significance of *A. abstrusus* in Israel, as well as a search for its endemic intermediate hosts. In the meantime, aelurostrongylosis should be included in the differential diagnosis of respiratory disease signs in domestic cats from Israel and its neighboring countries.

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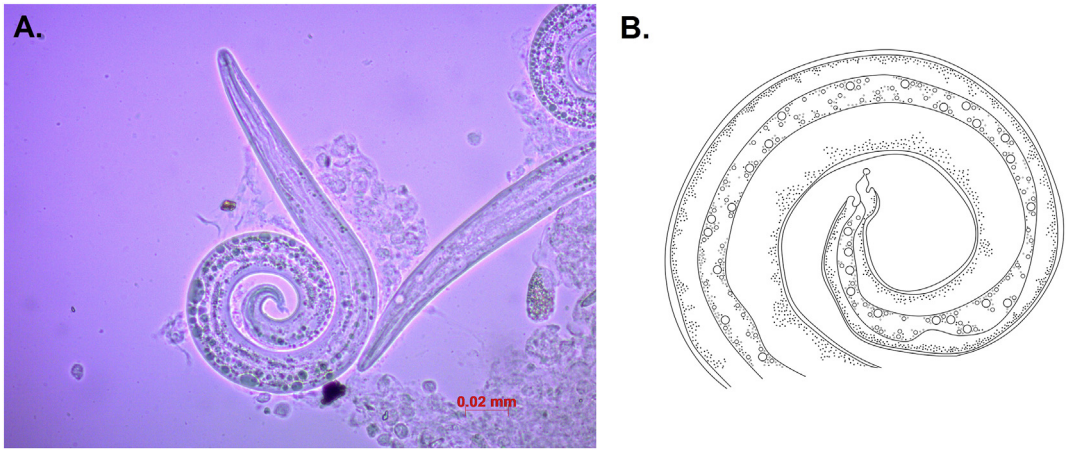

Fig. 2. (A) *Aelurostrongylus abstrusus* L1 larva in the bronchoalveolar lavage of the cat, observed under a light microscope. (B) A line drawing showing the posterior end of the larva with the characteristic S-shape and dorsal spine.

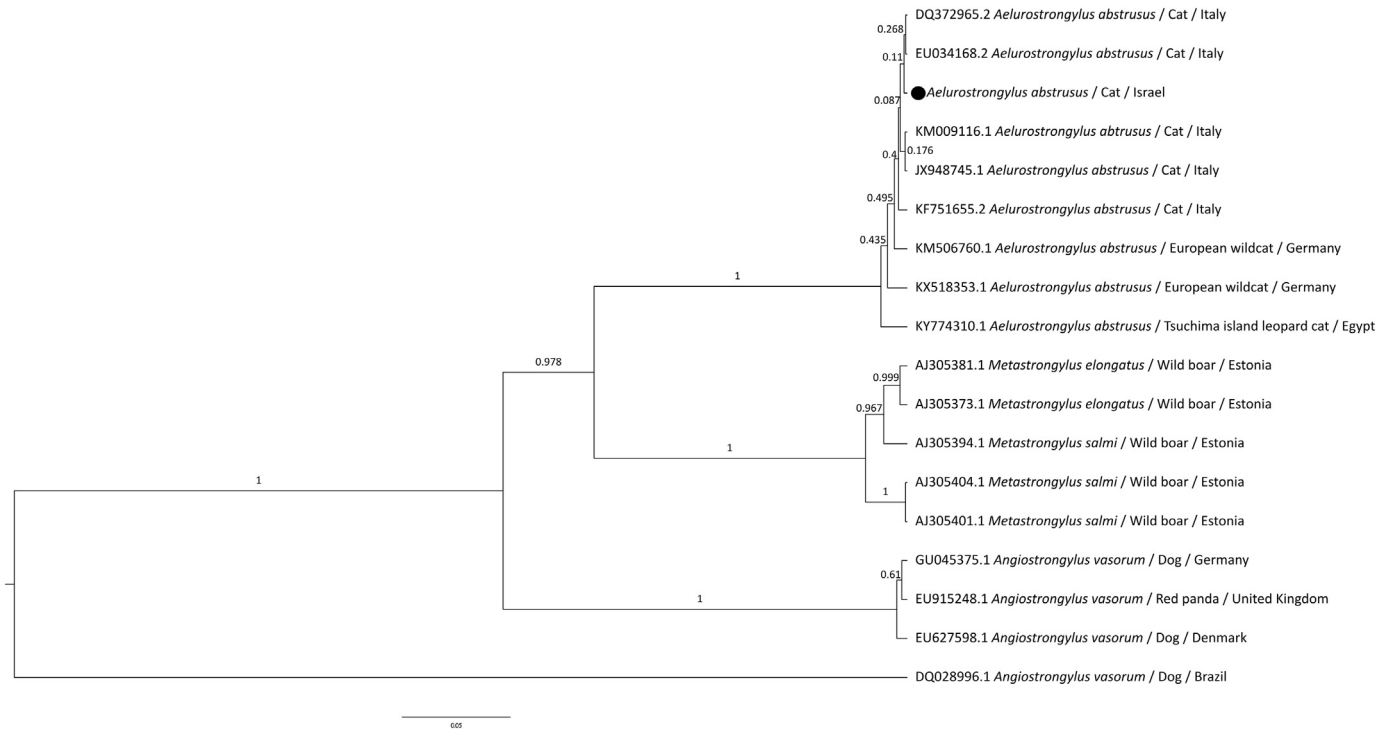

Fig. 3. Bayesian inference phylogenetic tree of an ITS-2 fragment of *A. abstrusus*. Posterior probabilities of each node are shown above each branch. The sequence obtained in the present study is marked with a black circle.

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Conflict of interest statement - competing interests

The authors declare no competing interests exist.

Ethical statement - animal welfare

No animal experimentation was done during the study described in this case report.

All tests were performed to the patient by its attending veterinary surgeons, as part of its clinical diagnosis and treatment, with the owners' informed consent.

Declarations of interest

None.

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