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Further studies of neuroangiostrongyliasis (rat lungworm disease) in Australian dogs: 92 new cases (2010–2020) and results for a novel, highly sensitive qPCR assay

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Abstract

The principal aim of this study was to optimize the diagnosis of canine neuroangiostrongyliasis (NA). In total, 92 cases were seen between 2010 and 2020. Dogs were aged from 7 weeks to 14 years (median 5 months), with 73/90 (81%) less than 6 months and 1.7 times as many males as females. The disease became more common over the study period. Most cases (86%) were seen between March and July. Cerebrospinal fluid (CSF) was obtained from the cisterna magna in 77 dogs, the lumbar cistern in f5, and both sites in 3. Nucleated cell counts for 84 specimens ranged from 1 to 146 150 cells μL^{-1} (median 4500). Percentage eosinophils varied from 0 to 98% (median 83%). When both cisternal and lumbar CSF were collected, inflammation was more severe caudally. Seventy-three CSF specimens were subjected to enzyme-linked immunosorbent assay (ELISA) testing for antibodies against *A. cantonensis*; 61 (84%) tested positive, titres ranging from <100 to ⩾12 800 (median 1600). Sixty-one CSF specimens were subjected to real-time quantitative polymerase chain reaction (qPCR) testing using a new protocol targeting a bioinformatically-informed repetitive genetic target; 53/61 samples (87%) tested positive, C_T values ranging from 23.4 to 39.5 (median 30.0). For 57 dogs, it was possible to compare CSF ELISA serology and qPCR. ELISA and qPCR were both positive in 40 dogs, in 5 dogs the ELISA was positive while the qPCR was negative, in 9 dogs the qPCR was positive but the ELISA was negative, while in 3 dogs both the ELISA and qPCR were negative. NA is an emerging infectious disease of dogs in Sydney, Australia.

Introduction

Canine neuroangiostrongyliasis (NA), or rat lungworm disease, is caused by migration of *Angiostrongylus cantonensis* or *A. mackerrasae* larvae through the nervous system (Barratt *et al.*, 2016). It was first reported in Brisbane in the 1970s (Mason *et al.*, 1976; Mason, 1987, 1989), with cases first seen in Sydney in 1990 (Collins *et al.*, 1992), increasing occurrence since then (Lunn *et al.*, 2003, 2012; Walker *et al.*, 2015) and a range extending as far south as Jarvis Bay and as far north as Townsville (Stokes *et al.*, 2007). The disease also occurs in horses (Wright *et al.*, 1991), zoo animals including primates (Carlisle *et al.*, 1998) and wildlife species such as parrots, tawny frogmouths (Reece *et al.*, 2013), possums (Ma *et al.*, 2013) and bats (Reddacliff *et al.*, 1999; Barrett *et al.*, 2002). NA follows the ingestion of snails and slugs, the intermediate hosts of *A. cantonensis* and *A. mackerrasae*, and potentially by ingestion of transport hosts such as planarians, centipedes, rodents and lizards (Mackerras and Sandars, 1955; Jindrak and Alicata, 1970; Bhaibulaya, 1975; Barratt *et al.*, 2016; Mendoza-Roldan et al., 2020). Third stage larvae (L_3) arrested in tissues of snails and transport hosts represent the infectious propagules for disease transmission (Bhaibulaya, 1975; Barratt *et al.*, 2016). First stage larvae (L_1) in rat feces are not infectious for dogs (Jindrak and Alicata, 1970; Barratt

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et al., 2016). The disease in Australia is most common in autumn, reflecting peak snail numbers. Young dogs are most frequently affected (Mason et al., 1976; Lunn et al., 2012).

The clinical presentation of NA is often syndromic (Mason, 1987; Lunn et al., 2012), especially in pups. Clinical signs develop approximately 10 days after snail ingestion (Jindrak and Alicata, 1970; Mason, 1987). Signs can be precipitated by macrocyclic lactones given during the prodromal period, if L_3 migrating through nerves, nerve roots and the spinal cord die rapidly and release foreign antigens (Lunn et al., 2012; Barratt et al., 2016). In pups, neuroanatomic features are typically lumbosacral and caudal at first, often with a mixture of upper and lower motor neuron signs in the back legs, with some tail weakness and variable incontinence (Mason et al., 1976; Collins et al., 1992; Lunn et al., 2012). Signs then ascend to become thoracolumbar and eventually cervical and cerebrocortical. Rarely, dogs can develop encephalitic NA without first demonstrating spinal signs. Marked hyperaesthesia is usually a prominent feature (Mason et al., 1976; Lunn et al., 2012), manifesting as spontaneous pain-causing abnormal neck carriage or a hunched posture, and sometimes aggressive behaviour after spinal palpation or manipulation of the neck or tail base (Barratt et al., 2016). In some cases, gentle stroking can elicit an aggressive response due to pain disproportionate to the stimulus (allodynia). Hyperesthesia presumably represents dermatomal neuropathic pain, arising from the involvement of peripheral nerves or nerve roots of the cauda equina (radicular NA) (Barratt et al., 2016). A presumptive diagnosis of canine NA is generally straightforward if the clinician is aware of this characteristic clinical picture.

A definitive diagnosis requires invasive confirmatory testing. A history of eating snails is helpful, but not always forthcoming. Peripheral eosinophilia is strongly supportive (Mason, 1989; Lunn et al., 2012), but also occurs with intestinal helminths and ectoparasites, especially fleas. Finding eosinophilic pleocytosis in cerebrospinal fluid (CSF) is strongly suggestive of NA, especially in Australia and Hawaii, but an unequivocal diagnosis can only be made by demonstrating larvae in CSF or by detecting *A. cantonensis* DNA in CSF using the polymerase chain reaction (PCR) (Qvarnstrom et al., 2016; McAuliffe et al., 2019) or next-generation sequencing (Xie et al., 2019; Zhang et al., 2020). A diagnosis of NA is strongly supported by demonstrating antibodies explicitly directed against *A. cantonensis* in CSF and/or serum. Serum is a less satisfactory sample because cross-reactions can occur with intestinal nematodes (Lunn et al., 2012). This issue has been addressed by new rapid immunochromatography methods (Eamsobhana et al., 2018; Eamsobhana et al., 2019; Somboonpatarakun et al., 2019; Somboonpatarakun et al., 2020) developed for human patients, using serum as the diagnostic specimen. As these tests detect only human antibodies, they are unsuitable for use in dogs without modification.

It would be helpful if a rapid point-of-care (PoC) test to diagnose NA was available for dogs using serum (ideally) or CSF. A rapid immunomigration test for detecting *A. cantonensis* antigen was developed (Chen et al., 2016), but is not commercially available and has therefore not been validated in dogs with NA. A similar PoC test exists for the detection of *Angiostrongylus vasorum* in dogs (Helm et al., 2010), using serum as the test sample (Schnyder et al., 2014). As *A. vasorum* is closely related to *A. cantonensis*, it is plausible that a kit designed to detect *A. vasorum* might also detect *A. cantonensis*. We tested CSF and serum from infected dogs and rats to evaluate this hypothesis. In addition, using archived and contemporaneously submitted CSF, we compared the accuracy of the enzyme-linked immunosorbent assay (ELISA) developed for human and canine patients at Westmead Hospital (Cross and Chi, 1982; Lunn et al., 2003) with a real-time quantitative PCR (qPCR) offered by Laverty Pathology Vetnostics and later with an improved qPCR developed at the National Institute of Allergy and Infectious Diseases (NIAID) in the USA. We sought to determine which test was most accurate at establishing a diagnosis of NA in dogs. An ancillary aim was to define further the epidemiology and clinical features of NA in an expanded cohort of dogs to consolidate and update earlier work (Lunn et al., 2012; Walker et al., 2015).

Materials and methods

Case recruitment, serum and CSF specimens

All dog serum and CSF specimens were obtained from patients suspected of having NA between 2010 and April 2020, based on history, signalment, clinical signs (paraparesis, hind limb proprioceptive ataxia, spinal and tail base hyperesthesia and urinary incontinence) and eosinophilic pleocytosis. Although the traditional definition of eosinophilic pleocytosis is taken as >10% eosinophils or >10 eosinophils μL^{-1} in CSF, we have broadened the definition in this study to include all dogs with consistent neurological signs and any eosinophils in cytocentrifuged CSF, as few other disease processes are associated with eosinophils in CSF in Australia. Where possible, serum was saved as well as CSF, so that serology (ELISA) could be performed on both specimens, ideally from samples collected simultaneously. CSF and serum specimens were archived and curated from various sources, including private veterinary pathology laboratories (Vetnostics, QML and IDEXX), university laboratories (Veterinary Pathology Diagnostic Services (VPDS), Sydney School of Veterinary Science (SSVS)) and the Parasitology Unit at Westmead Hospital, where samples had been sent for ELISA testing, typically with limited patient information. Samples were maintained at −4°C for a variable period (weeks–years) before thawing and testing using ELISA, PCR or antigen detection kits.

Serum and CSF were also obtained from rats with experimentally induced *A. cantonensis* infections. These rats comprised a small colony maintained to produce antigen for the serological diagnosis of NA. Animal ethics approval was granted from Western Sydney Local Health District/Westmead Animal Ethics Committee (Protocol number: 8003.03.18) to use rats that would otherwise be culled from the colony. Briefly, the life cycle of *A. cantonensis* was sustained in the Parasitology Unit, Institute of Clinical Pathology and Medical Research, Westmead Hospital using freshwater snails (*Biomphalaria glabrata*) infected by ingestion of plant material inoculated with infected rat feces. Wistar rats (*Rattus norvegicus*) were housed in the hospital vivarium. Feces containing L_1 (confirmed by Baermann technique) were collected from rats infected with *A. cantonensis*. Rats were given moderately severe chronic lungworm infections by feeding L_3 harvested from infected snails. After a prescribed time, usually 12 months, rats were euthanased, with blood and CSF collected under isoflurane anaesthesia immediately before euthanasia and necropsy. Infected rats showed only mild respiratory signs when initially infected, and readily produced large numbers of L_1 in their feces. Weight loss was the most notable long term finding in rats but was only seen in some individuals. Occasional rats were euthanased if they developed respiratory distress. At necropsy, female and male *A. cantonensis* adults were recovered from the right ventricle and both pulmonary arteries for antigen preparation.

Canine patients were investigated in a variety of different veterinary practices, typically multi-disciplinary referral centres, with samples (blood, serum &/or CSF) submitted to veterinary pathology laboratories, and then forwarded subsequently to Westmead Hospital for ELISA testing. Signalment, date of presentation and clinical data were obtained where possible using

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clinical pathology submission sheets and veterinary computerized records, when retrievable. For canine patients, CSF was concentrated by cytocentrifugation, stained with a rapid Romanowsky stain (e.g. Rapid-Diff) and examined using conventional light microscopy, while the total cell count was determined using an automated cell analyser. Some laboratories did a quantitative differential white cell count on CSF; others provided a qualitative report. Representative cytocentrifugation preparations of CSF from a dog with PCR-confirmed NA are presented in Fig. 1A.

ELISA testing

Antibodies against *A. cantonensis* antigens in CSF and serum from dogs were detected by an indirect ELISA (Lunn *et al.*, 2003, 2012) adapted from the method described by Cross and Chi (1982).

DNA extraction and qPCR testing

Initially, qPCR testing was conducted using a protocol developed in-house by Vetnostics laboratory. DNA extractions were performed using the NucleoMagR VET Viral RNA/DNA Isolation Kit (Macherey Nagel) on a Kingfisher Flex®. Briefly, 100 μL of CSF was combined with 30 μL of Molecular Grade water and 20 μL of 75 mg mL^{-1} Proteinase K (Macherey Nagel). If less than 100 μL of the sample was available, the balance was made up of PCR quality water. DNA was extracted out of the lysate during PCR testing, which included the cell suspension. The manufacturer's standard protocol was followed for extractions and resulted in an elution volume of 90 μL. Each batch of extractions included an extraction blank (PCR quality water instead of the sample) and positive control. *Angiostrongylus* qPCR was performed using primers AngFor 5′-GAATGCCACCTTGAATTGCTG-3′ and AngRev 5′-AACGCAAAACGTGCACACA-3′ from the 18S ribosomal RNA gene and 5.8S ribosomal RNA gene, using a FAM marker 5′FAM-TTGATAGTGCGTGTATGCA-MGB3′, purchased from Biolegio Gene Target Solution. The qPCR reactions were run in 25 μL volumes using Perfecta qPCR Toughmix (Biolegio Gene Target Solution) with primers at a final concentration of 600 nM and probe at 125 nM using 6 μL of DNA template. The assays were performed on Magnetic Induction Cycler thermocyclers (BioMolecular Systems). PCRs were initiated at 95°C for 5 minutes, followed by 40 cycles of 15 seconds at 95°C and 45 seconds at 63°C. Positive and negative controls were included for each reaction run. Positives were called if runs returned C_T values <38.

At the 6th International Workshop on *Angiostrongylus* and Angiostrongyliasis at Hilo in January 2020, we became aware of a novel qPCR developed at the NIAID using AcanR3990, a highly sensitive, bioinformatically-informed repetitive target (Sears *et al.*, 2020, submitted). This assay was subsequently utilized by VPDS. Briefly, total DNA was obtained from L_1 *A. cantonensis* SYD.1 isolated using faecal samples from an infected rat (Červená *et al.*, 2019; Valentyne *et al.*, 2020). DNA was isolated from a single L_1 and 3 L_1 that had been stored in 80% ethanol. Larvae samples were isolated with an Isolate II genomic DNA kit (Bioline, Australia) using the manufacturer's instructions for tissue samples, with overnight digestion in Proteinase K at 56°C. Total DNA was eluted in 50 μL of elution buffer and stored at −20°C. A fresh faecal sample (150 μg) from a rat infected with *A. cantonensis* SYD.1 was also processed. Total faecal DNA was isolated using the MagMAX™ CORE Nucleic Acid Purification Kit (complex protocol) with MagMAX™ CORE Mechanical Lysis Module & Glass Microbeads (Applied Biosystems, Thermo-Fisher Scientific, Australia) according to the manufacturer's instructions using the KingFisher Flex System (Thermo-Fisher Scientific, Australia). Feces in a tube with glass beads and lysis buffer were disrupted using a high-speed homogeniser, Mini-BeadBeater-96 (BioSpec Products, Daintree Scientific, Australia) at 2400 rpm for 40 seconds. Extracted DNA (90 μL) was stored at minus 20° C. Total DNA was isolated from archived CSF samples (50–200 μL) using an Isolate II genomic DNA kit (Bioline, Australia) according to the manufacturer's instructions utilizing protocol '9.5 Genomic/viral DNA from blood'. Total DNA was eluted in 50 μL of elution buffer and stored at −20°C. DNA was isolated in batches of 20 with blank controls, none of which indicated the presence of cross-contamination.

Angiostrongylus qPCR was run using primers (S0947, S0948) and probe (S0949) targeting AcanR3990 (William Sears *et al.*, 2020, submitted). This highly sensitive qPCR assay amplifies DNA of both *A. cantonensis* and *A. mackerrasae,* the 2 *Angiostrongylus* species endemic in rats in Australia (Červená *et al.*, 2019; Valentyne *et al.*, 2020). All primers and a fluorescently labelled probe (FAM/ZEN/3′IBFQ) were ordered from Integrated DNA Technologies Australia (Baulkham Hills, Australia). The qPCR reactions were run in 10 μL volumes using *Sso*Advanced Universal Probes Supermix (BioRad, Australia) with primers at a final concentration of 400 nM and probe at a final concentration of 100 nM. All samples were run in duplicate, using 1 μL of template DNA. The assays were performed in a CFX96 Touch Real-Time PCR Detection System with corresponding CFX Maestro 1.0 software (BioRad, Australia). PCRs were initiated at 95°C for 3 min, followed by 40 cycles of 10 s at 95°C and 20 s at 60°C. Each reaction contained DNA from 3 L_1 of *A. cantonensis* SYD.1 as a positive control (C_T value = 19), while ddH_2O acted as a negative control in each run. All runs were in duplicate and positives were called if both or one of the duplicate runs returned a C_T value <40.

Angio Detect™ antigen testing

The Angio Detect™ lateral flow immunoassay test developed by IDEXX Corporation for the rapid detection of *A. vasorum* antigen was designed for cage-side testing of dog serum, producing a result within 15 min (Schnyder *et al.*, 2014). We determined if this test would detect antigen of *A. cantonensis* (i.e. not the species for which the test was developed) in serum and/or CSF of dogs with NA, and in the serum of rats with moderate burdens of *A. cantonensis.*

Results

Case characteristics, annual occurrence and seasonality

Cases were recruited from North Shore Veterinary Specialist Centre (18 cases), Sydney Veterinary Emergency and Specialists (16 cases), Small Animal Specialist Hospital (10 cases), Veterinary Specialist Services (8 cases), University Veterinary Teaching Hospital Sydney (8 cases), Northside Veterinary Specialist Centre (4 cases) and Animal Referral Hospital (3 cases), with the remainder arising from veterinary pathology laboratories and private practitioners (Supplementary Table 1). Eleven of the cases were from Queensland (10 from Brisbane and 1 from Townsville), while the remainder were from Sydney, New South Wales.

Age was known for 90/92 dogs with NA and ranged from 7 weeks to 14 years [median age 5 months; interquartile range (IQR) 3–12 months]. The age distribution of affected dogs is illustrated in Fig. 2A, demonstrating 73/90 (81%) of dogs in the study cohort were younger than 6 months.

CSF was collected from all patients and was available for serological (ELISA) and/or qPCR testing in nearly all instances. Corresponding serum samples were also available for 7 patients.

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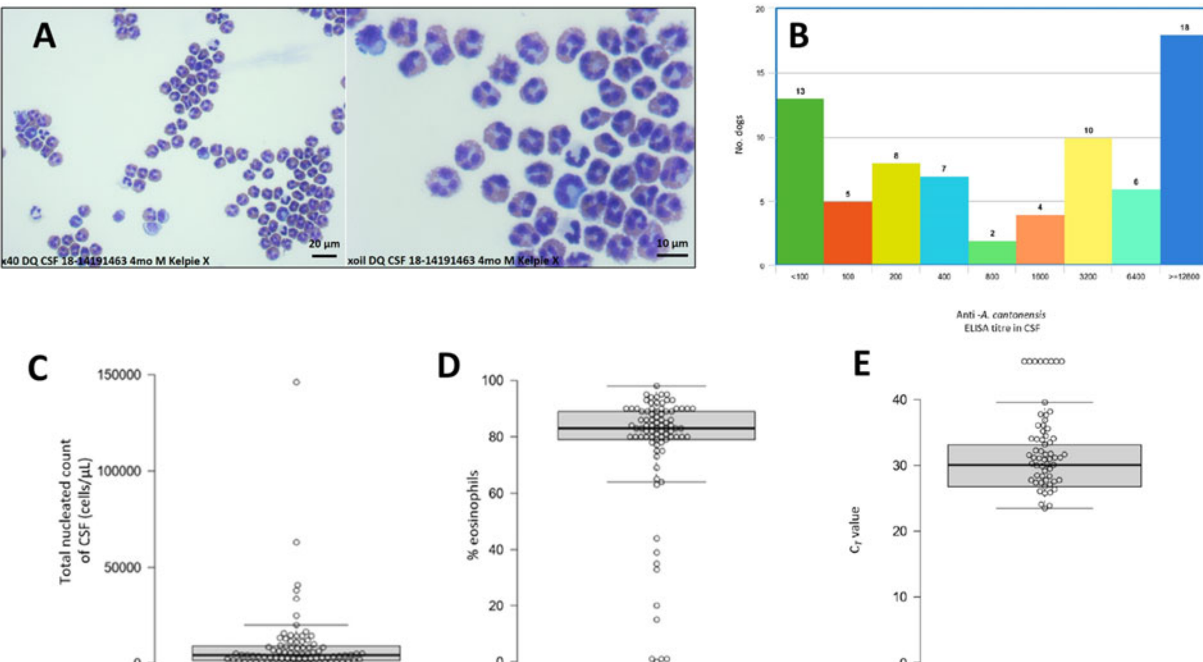

Fig. 1. (A) Cytocentrifuged sample of CSF from a 4-month-old male Kelpie crossbred (Case 52) with canine neuroangiostrongyliasis (NA). Note the cellularity of the specimen, with marked eosinophilic pleocytosis, evident in the 2 composite images provided at different magnifications. (B) Anti-*Angiostrongylus* cantonensis ELISA titres in CSF from 73 canine patients with NA. Antibody titre categories are provided along the *X*-axis, while the *Y*-axis shows the number of dogs having a given antibody titre. (C) Box-whisker plots of total nucleated cell count in CSF (cells/μL) for 84 dogs with neural angiostrongyliasis. The *Y*-axis is the nucleated cell count. In this and all similar plots, the box is the interquartile range (IQR), the median is the line within the box, and the 'whiskers' extend to data points that are less than 1.5 × IQR away from 1st/3rd quartile. CSF cytology data was irretrievable for 8 dogs. (D) Box-whisker plots of the percentage eosinophils in 85 specimens in which differential cell counts were retrievable. The *Y*-axis is the % eosinophils of the total nucleated cell count in CSF. (E) Box-whisker plots of the cycling threshold (C_T) values for the AcanR3990 qPCR for 61 CSF specimens from dogs with NA. The *Y*-axis displays the C_T values. The cut off for a positive C_T was taken to be 40. Note the 8 dogs that were qPCR negative appear at the top of the plots.

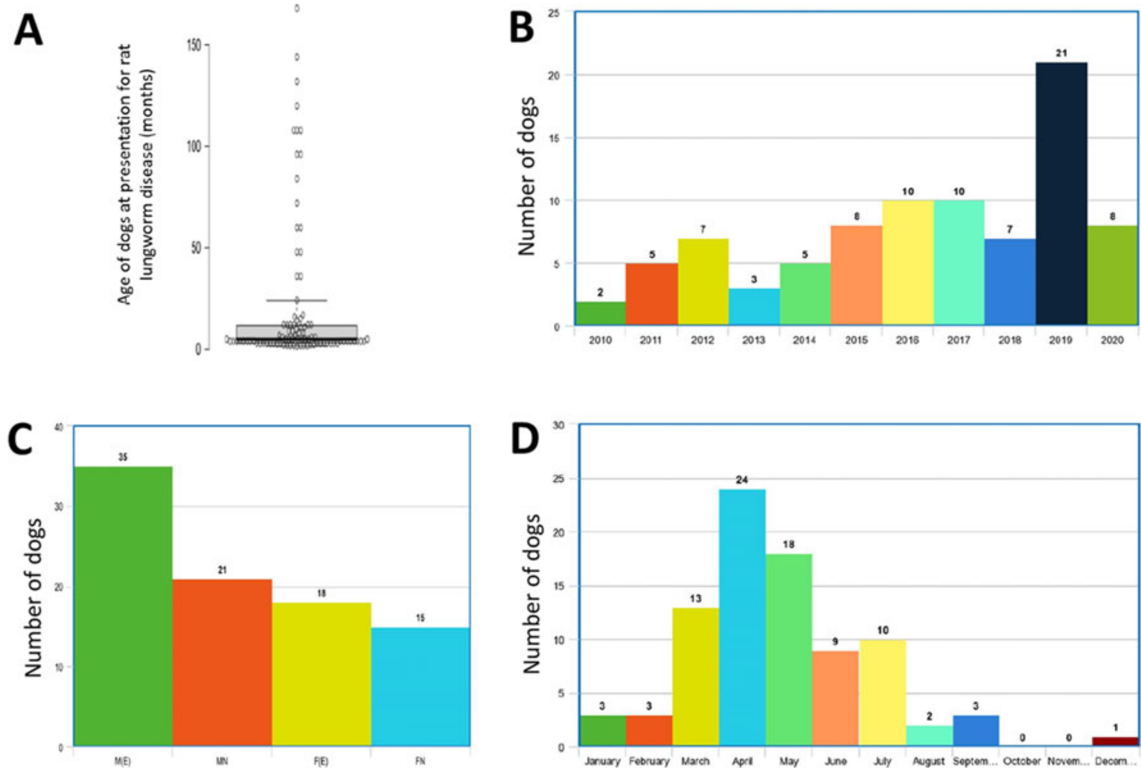

Fig. 2. (A) Box-whisker plot of the ages of 90 dogs with presumptive neural angiostrongyliasis. The *Y*-axis shows the age at presentation in months. Age data was irretrievable for two patients. (B) The number of retrievable cases of canine NA per annum during the study period. The *Y*-axis represents the number of dogs, while the year of presentation is given along the *X*-axis. The number of cases in 2020 was foreshortened because of the submission date for the paper and the impact of COVID-19 on veterinary practice, but additional 12 cases were seen between April and September 2020 (date of submission of the galley proofs). (C) Gender distribution of dogs with rat lungworm disease. The *Y*-axis shows the number of dogs, the *X*-axis the gender category: M(E) male entire, MN male neutered, F(E) female entire, FN female neutered. (D) The number of retrievable cases of canine NA per calendar month during the study period. The *Y*-axis is the number of dogs, while the month of presentation is given along the *X*-axis. Note that cases were encountered most often in autumn and winter.

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The 92 dogs comprised 19 Labrador retrievers, 8 Staffordshire Bull terriers (SBT) and 2 SBT crosses, 1 Poodle and 9 Poodle crossbreds (including 3 Groodles, 2 Spoodles and 1 Cavoodle), 5 French bulldogs, 4 Golden retrievers, 4 Miniature Schnauzers, 2 Jack Russel terriers (JRT) and 2 JRT crosses, 3 Rottweilers, 3 Boston terriers, 2 Australian and 1 American Bulldogs, 2 Border collies, 2 American Staffordshire Bull terriers, 2 Pugs, 2 German Shepherds, 1 Kelpie and 1 Kelpie cross, and one each of a wide variety of other breeds and crossbreds. No attempt was made to determine if certain breeds were overrepresented, although the breeds affected appeared to reflect their overall popularity, which changed over the 10-year study period.

The gender breakdown was 56 males (35 neutered; 21 entire) and 33 females (18 spayed; 15 entire), with gender irretrievable for 2 patients (Fig. 2C). There was thus a preponderance of male dogs. The higher than expected proportion of sexually entire dogs (36/89; 40%) likely reflects the tendency of this disease to occur before the age of surgical neutering.

For cases where the date of presentation was retrievable, 2 cases were seen in 2010, 5 cases in 2011, 7 in 2012, 3 in 2013, 5 in 2014, 8 in 2015, 10 in both 2016 and 2017, 7 in 2018, 21 in 2019 and 8 until April 2020 (Fig. 2B). An additional 10 cases were seen between April and August 2020, the date of submission for the revised paper. The date of presentation was irretrievable for 6 dogs. The trend was, therefore, for progressively more cases to be seen in successive years, culminating in a record annual occurrence in 2019.

The date of CSF collection was available for 86/92 patients, with 3 cases seen in January, 3 in February, 13 in March, 24 in April, 18 in May, 9 in June, 10 in July, 2 in August, 3 in September, none in October or November and 1 in December (Fig. 2D). Cases were mostly seen in autumn and winter.

CSF analysis

CSF was obtained from the cisterna magna in 78 dogs, from the lumbar cistern alone in 5 dogs and both sites in 3 dogs; the site was unrecorded for 6 cases. In the 3 dogs in which both cisternal and lumbar CSF specimens were collected, the nucleated cell counts in lumbar CSF (8340, 63 180 & 11 430) were substantially greater than the corresponding cisternal CSF specimens (1553, 3140 & 3860), respectively, indicating inflammation was more severe caudally. The highest cell count obtained from each dog was used for analysis.

Cell counts in CSF for the 84 specimens for which cell counts were retrievable ranged from 1 to 146 150 cells μL^{-1}, with a median of 4500 and an IQR of 1859.5 to 9195 cells μL^{-1} (Fig. 1C). Typically, CSF specimens were variably turbid, resembling coconut water in individuals with severe disease. The percentage eosinophils varied from 0% to 98% for the 85 specimens in which a differential cell count was performed, with a median value of 83% and IQR of 73–89% (Fig. 1D). Of the 85 dogs for which the differential eosinophil count in CSF was retrievable, all except 4 dogs had a 'traditional' eosinophilic pleocytosis. In the atypical patients (cases 7, 25, 48, 63), the total count was normal or only increased slightly, with the proportion of eosinophils low (215 and 15%; 45 and 33%; 2.2 and 0%; 1 and 1%), but all 4 were considered to have NA based on serology (case 7: ELISA titre 400) or qPCR data (cases 25, 48 & 63: C_T of 27.7, 23.8 & 27.7), respectively.

Case 48 was initially included amongst 5 CSF samples from dogs thought not to have NA. These dogs were included inadvertently for qPCR testing and thus initially considered as control samples. Four of the dogs were negative on qPCR testing, as expected. One dog, surprisingly, was positive, with a C_T of 23.8; this patient was a 5-year-old French bulldog presented for sudden onset head tremor. It was initially diagnosed with idiopathic head tremor syndrome (Guevar *et al.*, 2014), but as CSF analysis and MRI findings were considered unremarkable, it was reassigned as having NA based on the qPCR result.

ELISA serology

It has been shown that in normal pounds dogs (i.e. negative controls), the anti-*A. cantonensis* ELISA titres of CSF samples were always less than 100 unless CSF was contaminated by the blood during collection (Lunn *et al.*, 2003, 2012). In contrast, serum specimens from a substantial proportion of young normal pound dogs can have ELISA titres as high as 6400, presumably due to cross-reacting antibodies directed against gastrointestinal nematodes (Lunn *et al.*, 2003, 2012). Thus, CSF antibody titres of ⩾100 and serum antibody titres of >6400 were classed as positive.

Considering CSF specimens from NA cases subjected to ELISA testing for antibodies against *A. cantonensis*, 60/73 (82%) tested positive (Fig. 1B). Eighteen dogs had CSF titres greater than or equal to 12 800. Of 13 CSF specimens deemed to be ELISA negative (at a single point in time), 9 were qPCR positive, 3 were also qPCR negative and 1 had unknown qPCR status, but all were considered to have NA based on eosinophilic pleocytosis, characteristic clinical and/or imaging findings and response to treatment.

Simultaneous serum ELISA titres were available for 7 patients, with titres ranging from 200 to ⩾12 800. The actual titres were 200, 400, 400, 400, 800, 800 and ⩾12 800. The serum titre was lower (4 specimens), equal to (2 specimens) or higher (1 specimen) than the titre in the corresponding CSF specimen (supplementary Table 1). Therefore, the serum titre was high enough to diagnose NA in just 1 dog.

Real-time PCR data

A total of 73 CSF specimens were subjected to qPCR testing. Of the 25 CSF specimens tested using the Vetnostics qPCR assay, only 8 (32%) were positive. Of 61 CSF specimens tested with the AcanR3990 qPCR assay (including 14 samples tested previously using the Vetnostics assay), 53 (87%) tested positive (Fig. 1E). The median C_T value was 30.0, with an IQR of 26.7 to 33.1. Some C_T values may have slightly underestimated the amount of DNA present (C_T higher than expected), as the volume of CSF available for testing was sometimes 50 or 100 μL, rather than 200 μL, due to scarcity of sample.

Considering the 13 CSF specimens tested using both PCR assays; in 11 instances the AcanR3990 qPCR was positive (C_T ranging from 27.3 to 39.5) while the Vetnostics assay registered as negative, in 1 case both qPCR assays were negative, and both assays were positive for 2 specimens, but with the C_T value being substantially lower for the AcanR3990 qPCR (26.3 *v* 32.4; 24.0 *v* 30.9).

Of the 8 CSF specimens that were qPCR-negative using the AcanR3990 assay, 5 were ELISA-positive for antibodies. The remaining 3 samples were ELISA-negative but still considered to have NA based on clinical signs, CSF and/or MRI findings and subsequent response to therapy.

In 2 dogs whose CSF was AcanR3990 qPCR-positive, blood and urine were also tested, but no nucleic acid could be detected in these samples. Of 7 serum specimens from rats chronically infected with moderate *A. cantonensis* burdens, only 1/7 tested qPCR positive with the Vetnostics PCR protocol, with a C_T value of 31; in this instance, blood had been collected by cardiac puncture immediately after euthanasia, so impaling an adult *A. cantonensis* worm, larvae or eggs during the procedure was a possible explanation for the single positive qPCR result (Table 1).

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Table 1. Results of *A. vasorum* point-of-care antigen test in rats chronically affected by moderate burdens of *A. cantonensis*

Experimental infections in adult Wistar rats	Adult worm burden in pulmonary arteries and right ventricle	Sample	PCR result/C_T value	IDEXX Angio Detect™
Rat 1	10 males 13 females 23 total	Serum	negative using Vetnostics PCR	negative
Rat 2	4 males 4 females 8 total	Serum	negative using Vetnostics PCR	negative
Rat 3	3 males 10 females 13 total	Serum	negative using Vetnostics PCR	negative
Rat 4	12 males 7 females 19 total	Serum	negative using Vetnostics PCR	negative
Rat 5	1 male 5 females 6 total	Serum	negative using Vetnostics PCR	negative
Rat 6	13 males 10 females 23 total	Serum	negative using Vetnostics PCR	n/a
Rat 7	11 males 13 females 24 total	Serum	positive C_T 31* using Vetnostics PCR	n/a

*The blood was collected by cardiac puncture and likely the needle would have traversed lung tissue to reach the heart; the lungs would have contained *A. cantonensis* larvae and eggs; thus the blood and the harvested serum may have been contaminated by this process.

A. vasorum antigen testing of CSF and rat serum

The Angio Detect™ test was run on 6 canine CSF specimens from dogs with NA (Table 1). All tested negative. The same assay was run using serum from 5 rats chronically infected with moderate burdens of *A. cantonensis* (Table 1); all 5 samples tested negative.

Discussion

This study confirms and extends our understanding of canine NA in Australia. There are 2 new findings concerning the epidemiology of rat lungworm disease in dogs. Firstly, the condition would appear to be increasingly prevalent, with 21 new cases in 2019 alone and a similar trend for 2020. This suggests that NA is an emerging infectious disease of dogs in Sydney (Collins *et al.*, 1992; Lunn *et al.*, 2003, 2012; Walker *et al.*, 2015). The simultaneous emergence of a cluster of leptospirosis cases in dogs of inner Sydney in 2019/20 suggests that increased numbers of rats or increased dispersal of rats because of light rail construction might have contributed to this phenomenon (Zhou, 2019). Secondly, the preponderance of male dogs amongst affected individuals with NA is apparent from this much-expanded dataset, perhaps reflecting an increased propensity for male dogs and puppies to explore and sample their environment. In accord with earlier work, young dogs (81% less than 6 months of age) were most frequently affected, presumably because of their inquisitive nature and tendency to chew and eat indiscriminately, and perhaps because they have not started long-acting heartworm prophylaxis, which might kill ingested L_3 (Mason, 1987; Collins *et al.*, 1992; Lunn *et al.*, 2012; Walker *et al.*, 2015). Interestingly, the first report of *Angiostrongylus costaricensis* infection in a dog was also in a puppy (Alfaro-Alarcón *et al.*, 2015).

The disease in dogs has a shorter prodrome than in people, with clinical signs typically occurring 10 days after ingestion of infective L_3 (Jindrak and Alicata, 1970; Mason, 1987), whereas in human patients the lag phase can extend to 3 weeks (Blair *et al.*, 2013; Morton *et al.*, 2013; Ming *et al.*, 2017; Ansdell and Wattanagoon, 2018; Busse *et al.*, 2018; Prociv and Turner, 2018; Berkhout *et al.*, 2019; McAuliffe *et al.*, 2019). Of human patients, children and especially infants are more likely to have severe disease, with greater morbidity and higher mortality and a tendency to suffer long-term neurologic sequelae (Morton *et al.*, 2013; Prociv and Turner, 2018; Berkhout *et al.*, 2019). This might be extrapolated to smaller canine patients especially puppies, as a severe disease may reflect (i) a relatively larger dose of L_3 in relation to the size of the brain, spinal cord, nerves and nerve roots and (ii) a less 'experienced' immune response.

Most dogs that developed NA have been pedigree dogs or pedigree dog hybrids, generally reflecting the changing trends of breed popularity, for example, the increased occurrence of small brachycephalic dogs amongst the study cohort in recent years (McGreevy and Fawcett, 2019). The appearance of cases in autumn and winter is consistent with earlier reports and reflects these as peak times for the occurrence of snails in the accessible environment (Mason *et al.*, 1976; Collins *et al.*, 1992; Lunn *et al.*, 2012; Walker *et al.*, 2015). Most cases in our dataset were from dogs residing in Sydney rather than Brisbane or other coastal Queensland cities, which was surprising considering the disease has been considered endemic in south-east Queensland since the 1970s and the warmer, wetter, subtropical environment might be expected to favour a larger snail population. It might be that global warming has decreased spring rain in Brisbane to the extent that snails are less prevalent than in the past, although this should be explored by fieldwork and analysis of meteorological records.

Historically, the gold standard for the diagnosis of NA in human patients was macroscopic and microscopic examination of the brain and spinal cord at necropsy, or identification of L_3 in CSF or aqueous humour (in ocular angiostrongyliasis) (Barratt *et al.*, 2016). Positive amplification of *A. cantonensis* DNA from CSF is also definitive (Blair *et al.*, 2013; Morton *et al.*, 2013; Qvarnstrom *et al.*, 2016; Ming *et al.*, 2017; Ansdell and Wattanagoon, 2018; Busse *et al.*, 2018; Prociv and Turner, 2018; Berkhout *et al.*, 2019; McAuliffe *et al.*, 2019), although at some stages of infection there may be insufficient parasite DNA in CSF to permit a diagnosis by this means (Qvarnstrom *et al.*,

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2016). Detecting anti-*A. cantonensis* antibodies in CSF, serum or blood using immunoassays can also provide a definitive diagnosis of NA (Cross and Chi, 1982; Eamsobhana *et al.*, 2018; Eamsobhana *et al.*, 2019; Somboonpatarakun *et al.*, 2019, 2020), although it is sometimes necessary to sample CSF on more than one occasion to obtain a positive result, as the host immunoglobulin response is time-dependent (Blair *et al.*, 2013; Morton *et al.*, 2013; Barratt *et al.*, 2016; Ming *et al.*, 2017; Ansdell and Wattanagoon, 2018; Busse *et al.*, 2018; Prociv and Turner, 2018; McAuliffe *et al.*, 2019). The performance of serological immunoassays has been improved by the development of recombinant antigens (Somboonpatarakun *et al.*, 2019, 2020) and other methodological refinements (Eamsobhana *et al.*, 2018; Eamsobhana *et al.*, 2019). The availability of these immunoassays as rapid PoC tests (Eamsobhana *et al.*, 2018, 2019; Somboonpatarakun *et al.*, 2019, 2020) greatly facilitates diagnosis of NA in human patients in endemic regions such as Thailand. Unfortunately, the modern PoC lateral flow tests do not currently detect canine immunoglobulins and the lateral flow rat lungworm antigen test from China (Chen *et al.*, 2016) is not yet distributed commercially.

The present study attempted to address similar issues concerning diagnosis in a large cohort of canine patients likely to have NA. In experimentally induced canine NA (Jindrak and Alicata, 1970; Mason, 1989), dogs develop clinical signs approximately 10 days after L_3 ingestion. During this latent period, antigens and nucleic acid in excretory products shed cuticle and dead and degenerating larvae are generated as the L_3 make their way from the gut to the central nervous system (CNS), where extensive larvae growth, migration and moulting occur. Presumably, this is sufficiently long for the immune system to develop IgM and IgG against larval antigens. Unfortunately, testing for both antibodies and nucleic acid was not possible for all patients in the study cohort. However, for 57 CSF specimens, we had a robust dataset with both ELISA serology and results of the novel qPCR available for systematic comparison.

Of the CSF specimens tested with the AcanR3990 qPCR, 53/61 (87%) tested positive. Of the 73 CSF specimens subjected to ELISA testing for antibodies against *A. cantonensis*, 60/73 (82%) tested positive. Considering just the 57 specimens for which both ELISA and AcanR3990 qPCR data were available, 49/57 (86%) were qPCR positive, while 45/57 (79%) were ELISA positive. There was therefore general agreement that in a canine cohort, the qPCR had a sensitivity of almost 90%, while the ELISA had a sensitivity of approximately 80%. In contrast, the initial Vetnostics qPCR had a sensitivity of only 32%. If AcanR3990 qPCR and ELISA results were considered in concert, 54/57 (95%) of NA cases could be confirmed using a single CSF sample.

Thus, to confirm a presumptive diagnosis of canine NA, it is ideal to undertake both qPCR and ELISA testing. A positive result using qPCR and/or serology on CSF is sufficient to confirm a diagnosis of NA, whereas a negative result for either or both does not exclude rat lungworm disease, as a given specimen might be collected before sufficient antibody or nucleic acid has been released into the CSF (Barratt *et al.*, 2016; Qvarnstrom *et al.*, 2016). There were 3 dogs whose CSF was negative for antibodies and nucleic acid using ELISA and qPCR testing but in other respects had typical findings of NA; collection and testing of a second CSF specimen at a later time could well have proved to be positive in these patients, although owners would understandably be reluctant to permit (and pay for) such further testing, especially if the dogs had responded to preliminary empiric therapy. In human patients, enough DNA for detection using qPCR is not always present in CSF at the time symptoms first develop, and thus obtaining sequential CSF samples may be required to secure a diagnosis (Barratt *et al.*, 2016; Qvarnstrom *et al.*, 2016).

Considering the AcanR3990 qPCR data in dogs, the median C_T value was 30.0, with an IQR of 26.7 to 33.1 and a total range of 23.4 to 39.5. Thirty-one of 53 CSF specimens had a C_T over 30, despite PCR primers being directed at a highly sensitive, bioinformatically-informed repetitive target within the *A. cantonensis* genome. This compares to the original PCR assay developed by Qvarnstrom and colleagues at the Centre for Disease Control (CDC) targeting ITS1, where C_T values for human CSF specimens varied from 27 to 38, with 20/24 being over 30 (Qvarnstrom *et al.*, 2016).

Currently, there is insufficient evidence as to whether CSF collected from the lumbar cistern is superior to CSF collected from the cisterna magna for laboratory testing. In 3 patients where a comparison could be made between cisternal and lumbar samples, inflammation was more pronounced caudally. It is interesting that in human patients, sitting upright for several hours results in a higher likelihood of larvae appearing in CSF specimens collected by a lumbar tap. It should be acknowledged, however, that contamination of CSF with blood is more likely with lumbar *vs* cisternal taps in dogs.

There is consensus in the human literature that validated qPCR testing of CSF in human adults and infants with NA is usually fruitful diagnostically (Blair *et al.*, 2013; Morton *et al.*, 2013; Barratt *et al.*, 2016; Qvarnstrom *et al.*, 2016; Ming *et al.*, 2017; Ansdell and Wattanagoon, 2018; Busse *et al.*, 2018; Prociv and Turner, 2018; Berkhout *et al.*, 2019; McAuliffe *et al.*, 2019; Sears *et al.*, submitted), with 32/49 (65%) CSF specimens from 33 patients with NA testing positive in the most extensive study to date (Qvarnstrom *et al.*, 2016). Some successive specimens from the same patients had discrepant results, with a tendency for negative results to occur early in the disease course, as might be expected from the pathophysiology. Cellular debris from A. cantonensis released due to moulting and larval death is released into the subarachnoid space, elaborating DNA that forms the template for the qPCR reaction. In the original CDC qPCR, a single L_3 contained enough DNA template to be positive after 23 PCR cycles (i.e. C_T value of 23, on average). C_T values for qPCR-positive CSF specimens from human NA patients ranged from 27 and 38, i.e. 1–4 $\log_{10}$ less DNA than present in a single L_3. Thus, the qPCR presumably detects DNA from larval remnants, such as individual cells, nuclei or chromosome fragments that leak into the CSF from the brain and spinal cord parenchyma.

What came of a complete surprise to us was the observation that the qPCR could be positive in dogs with the neurological disease but normal CSF (no eosinophils!) and an unremarkable MRI scan (case 48), or in which the eosinophil count did not reach the traditional threshold for eosinophilic pleocytosis (cases 7, 25, 63). Furthermore, in 3 of these 4 cases, ELISA serology was negative. Presumably, the qPCR may pick up parasite nucleic acid during early stages of infection before an inflammatory response is well developed. For this reason, it would be prudent to incorporate the AcanR3990 qPCR into a multiplex panel including all the key canine neuropathogens. This would ensure all canine patients with the neurologic disease are screened for NA, even when characteristic clinical signs are absent.

In terms of non-invasive diagnosis, in only 1/7 serum specimens from dogs with NA was the ELISA titre sufficiently high ($\geqslant$12 800) to confirm a presumptive diagnosis of NA without resorting to CSF collection. A 15% success rate using serum as the diagnostic specimen for ELISA testing (as opposed to CSF) is an unsatisfactory proposition for most owners. Yet, a 5-year-old Labrador retriever with a serum ELISA titre of $\geqslant$12 800 not included in this study (due to the absence of confirmatory CSF data) probably had NA based on the extent of serum ELISA titre elevation. Adaptation of the latest generation of human

lateral flow assays (Eamsobhana *et al.*, 2018, 2019; Somboonpatarakun *et al.*, 2019, 2020) so that they will detect canine immunoglobulins might greatly improve the sensitivity and specificity of diagnostic serology in dogs with NA and even permit rapid, non-invasive diagnosis using a blood sample in a proportion of patients.

The same might be true of the lateral flow tests that detect *A. cantonensis* antigen in serum which we have been unable to source from its developers in China (Chen *et al.*, 2016). This rapid test has not been validated in dogs, although a similar test exists for the detection of canine infections with the related metastrongylid pathogen *Angiostrongylus vasorum*, the causative agent of 'French heartworm disease' (Helm *et al.*, 2010). This infection has a similar lifecycle to *A. cantonensis*, with the dog as the definitive host, adult worms living in the right ventricle and pulmonary arteries, and L_1 appearing in the feces (following ingestion of expectorated larvae) (Helm *et al.*, 2010). The Angio Detect™ PoC test detects a specific antigen elaborated by the parasite using serum as the diagnostic specimen (Schnyder *et al.*, 2014). As *A. vasorum* is closely related to *A. cantonensis*, it seemed a reasonable proposition that a kit designed to detect excretory antigens of *A. vasorum* (Schnyder *et al.*, 2014) might also detect those of *A. cantonensis*. The Angio Detect™ test was used on 6 CSF specimens from dogs with NA, but 6/6 tested negative. To determine if the issue was insufficient antigen as opposed to antigen dissimilarity, we examined the serum of rats with chronic lungworm infections. Testing the serum of rats with patent *A. cantonensis* infections is akin to testing the serum of dogs infected with *A. vasorum*, the species for which the kit was validated. As all 7 rat serum samples tested negative, the antigens elaborated clearly did not react with the capture antibodies in the kits. Such testing, therefore, has no place in diagnosing NA in dogs.

Conclusions

Canine NA is becoming more common as a cause of neurologic disease in eastern Australia, especially in Sydney and particularly in young dogs, rivalling more established entities such as meningitis of unknown origin (formerly granulomatous meningoencephalitis), aseptic suppurative meningitis, cryptococcosis, toxoplasmosis and neosporosis (Parzefall *et al.*, 2014) as causes of inflammatory CNS disease. In the UK and Europe, ectopic *A. vasorum* infections can give rise to neurologic signs associated with eosinophilic pleocytosis in dogs, although such reports are currently rare (Negrin *et al.*, 2008; Alcoverro *et al.*, 2019). Veterinarians have a role to play in recommending measures that safely control rodent and mollusc numbers in urban and suburban settings, while the regular monthly administration of products containing the long-acting macrocyclic lactone moxidectin is likely to be effective against the development of NA based on extrapolation from *A. vasorum* prophylaxis (Helm *et al.*, 2010). Should a dog be seen to eat a slug, snail or rat, immediate pre-emptive therapy using a 5-day course of fenbendazole or a single dose of moxidectin is prudent to prevent L_3 reaching the CNS. The same advice is relevant to children and infants (The Sydney Children's Hospitals Network, 2018; Children's Health Queensland Hospital and Health Service, 2019; NSW Health, 2019). As long-acting moxidectin (as a depot formulation) is a popular strategy for heartworm prevention in Sydney, the annual injection of ProHeart 12™ (McTier *et al.*, 2019) could be timed to occur in early autumn so that maximum blood levels are present in autumn and early winter when the risk of developing NA is highest.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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