Using cerebrospinal fluid to confirm Angiostrongylus cantonensis as the cause of canine neuroangiostrongyliasis in Australia where A. cantonensis and Angiostrongylus mackerrasae co-exist

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ABSTRACT

Both Angiostrongylus cantonensis and Angiostrongylus mackerrasae have been identified along the east coast of Australia. A lack of A. mackerrasae genomic data until 2019, however, has precluded the unequivocal identification of the Angiostrongylus species responsible for neuroangiostrongyliasis in accidental hosts such as dog and man. The availability of a whole-genome data for A. mackerrasae, including mtDNA and ITS2 rDNA, enables discrimination of A. cantonensis from A. mackerrasae. The aim of this study was to develop diagnostic PCR assays to determine the species of Angiostrongylus based on the detection of Angiostrongylus DNA sequences in the cerebrospinal fluid (CSF) of canine patients with eosinophilic meningitis. An in silico workflow utilising available cytochrome c oxidase 1 (cox1) primers streamlined the laboratory work into empirical steps, allowing optimisation and selection of a PCR assay that met the required criteria for discrimination of A. cantonensis and A. mackerrasae DNA in low-template CSF samples. The adopted cox1 qPCR assay specifically amplified and enabled the differentiation of A. cantonensis from A. mackerrasae DNA and confirmed the presence of A. cantonensis DNA in 11/50 archived CSF samples. The DNA sequences demonstrated the presence of two distinct A. cantonensis cox1 haplotypes in dogs from eastern Australia. Species identification was further confirmed via the adoption of an ITS2 rDNA assay, providing confirmation of only A. cantonensis ITS2 rDNA in the CSF samples. To our knowledge, this is the first study to unequivocally demonstrate the antemortem presence of A. cantonensis DNA in CSF from clinically affected dogs. The study confirmed the long-held assumption that A. cantonensis is the causal agent of neuroangiostrongyliasis but refutes the dogma that there was a single introduction of A. cantonensis into Australia by the demonstration of two distinct A. cantonensis cox1 haplotypes.

1. Introduction

The rat lungworm (Angiostrongylus cantonensis) is a nematode parasite of rats and gastropod molluscs known to accidently infect humans, dogs, and other animals where it causes eosinophilic meningitis and neuroangiostrongyliasis (NA) (Wang et al., 2008; Lee et al., 2021). Alongside its definitive hosts, rats in the genus Rattus, A. cantonensis has invaded many regions of the world including Australia (Mackerras & Sandars, 1955; Tokiwa et al., 2013; Rodpai et al., 2016). Humans are exposed to A. cantonensis via the consumption of snails and slugs, or by the ingestion of paratenic hosts such as crabs, prawns and planarians (Wang et al., 2008). Once ingested, larval stages of A. cantonensis have an obligatory migration through the central nervous system (CNS), where a florid immunological response in accidental hosts leads to syndromic clinical presentations (Murphy & Johnson, 2013).

Numerous cases of NA have been recorded in humans and animals along the east coast of Australia over the past 50 years (Bhaiyulaya, 1968; Wang et al., 2008; Diao et al., 2011; Lunn et al., 2012; Murphy & Johnson, 2013), with the most renowned case being that of a Sydney teenager who was infected after eating a slug on a dare (Senanayake et al., 2003). In...
Australia, dogs are much more commonly infected with *Angiostrongylus* spp. than people, and yet there are limited methods available for the diagnosis of canine NA. Diagnosis is contingent on the invasive collection of cerebrospinal fluid (CSF) and demonstration of eosinophilic pleocytosis (Lunn et al., 2003; Lee et al., 2021). Further confirmation requires either ELISA testing for anti-*A. cantonensis* antibodies and, more recently, the confirmation of *Angiostrongylus* spp. DNA via qPCR (Lee et al., 2021).

As a generalisation, NA is assumed to be caused by infection with *A. cantonensis* (Barratt et al., 2016). In Australia, however, two *Angiostrongylus* species – the invasive emerging *A. cantonensis* and the native *A. mackerrasae* – are known to be neurotropic in their definitive rat hosts (Bhabulaya, 1974; Cervená et al., 2019; Valentine et al., 2020). A lack of methods enabling species differentiation has prevented the definitive confirmation or exclusion of *A. mackerrasae* as a cause of clinical disease in dogs. At least one report using morphology demonstrated the presence of *patent A. mackerrasae* as the cause of angiostrongyliasis in a black flying fox (Mackie et al., 2013). Aside from rare instances where necropsy material has enabled species differentiation, the causative agent of NA in dogs in Australia remains unresolved (Lunn et al., 2012; Lee et al., 2021).

Recently, the species status of *A. cantonensis* and *A. mackerrasae* was reconfirmed using complete mitochondrial (mt) DNA, where a 7.1–7.5% difference between the two was demonstrated (Valentine et al., 2020). The molecular discrimination of *Angiostrongylus* spp. commonly employs mtDNA, with many studies targeting cytochrome c oxidase 1 (cox1) from either adult specimens or larval stages within gastropod molluscs (Tokiwa et al., 2013; Lv et al., 2017, 2018). We hypothesise that the relative abundance of cox1 template DNA available for amplification, combined with the significant nucleotide difference between *A. cantonensis* and *A. mackerrasae* (~10%), may enable the differentiation of *Angiostrongylus* spp. cox1 DNA sequences in cerebrospinal fluid (CSF) in suspected cases of canine NA and hence confirm the causative agent of disease.

The aim of this study was to adapt existing primers to develop a PCR assay that can determine the species of *Angiostrongylus* present in the CSF of canine patients based on the detection of mtDNA. To do so, we utilised archived and curated CSF samples from canine NA cases from Australia (Lee et al., 2021). The PCR assay targeted the highly variable cox1 region, which is able to discriminate reference *A. cantonensis* DNA from that of *A. mackerrasae* (Valentine et al., 2020). The cox1 DNA sequence results were further verified by the adoption of a nuclear marker enabling DNA sequence analysis of ITS2 rDNA assay, which was capable of amplifying both *A. cantonensis* and *A. mackerrasae*.

2. Materials and methods

2.1. Samples

DNA previously extracted from *A. cantonensis* (SYD.1, 10 ng/μl) and *A. mackerrasae* (ANWC:N5721 - P43/19-E, 2 ng/μl) were used throughout the study as positive controls, while DNA from archived CSF samples of 61 dogs from eastern Australia with eosinophilic meningitis (DOG 1-61, 50 cycles) were made up to 30 μl including 2 μl of template DNA, and were performed using MyTaq™ Red mix (Bioline, Australia), distilled water and primers at a concentration of 0.33 μM. Each PCR run included a no template negative control (distilled water). Cycling conditions used were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 52°C for 15 s and 72°C for 20 s, with a final extension step at 72°C for 7 min. Selected cox1 assays capable of amplifying the highest dilution of *A. cantonensis* DNA (0.2 pg/μl) were then optimised using a temperature gradient from 52°C to 62°C, as described above, to decrease the presence of non-specific binding and primer dimers. Finally, the selected assays were tested on *A. mackerrasae* DNA isolated from an adult worm and dog DNA to determine the ability of the primer pairs to amplify both species. Two additional known-positive canine CSF samples (see Section 2.1) were included to ensure their capacity to detect *Angiostrongylus* spp. DNA in the presence of canine eosinophils. PCR products were visualised on a 2% agarose gel stained with GelRed (Biotium) and observed under UV-light.

2.2. In silico selection of cox1 primers to amplify *A. cantonensis* and *A. mackerrasae*

Mitochondrial markers from complete Australian reference mtDNA genomes of *A. cantonensis* SYD. 1 (GenBank: MK570631) and *A. mackerrasae* P43/19-E (GenBank: MN793157) were used to compare the similarity of mitochondrial regions (Cervená et al., 2019; Valentine et al., 2020). All cox1 primers from previous studies on *Angiostrongylus* parasites were collated and tabulated (Monte et al., 2012; Tokiwa et al., 2012; Moreira et al., 2013; Nakaya et al., 2013; Okano et al., 2014; Apichat et al., 2016; Rodpai et al., 2016; Eamsobhana et al., 2017; Cervená et al., 2019; Valentine et al., 2020). Primers were mapped onto the reference sequences of *A. cantonensis* SYD. 1 (MK570631) and *A. mackerrasae* P43/19-E (MN793157) and the number of mismatches and their position relative to the reference mtDNA genomes were recorded before a combination of forward and reverse primers were selected for testing. Primers met the following criteria: (i) amplification of a short sequence (< 300 bp) that (ii) enabled species differentiation between *A. cantonensis* and *A. mackerrasae* with (iii) ≤ 3 mismatches between the primer and template DNA sequence (Table 1).

2.3. Partial cox1 PCR assay selection for the detection of *A. cantonensis* and *A. mackerrasae* DNA

Assays selected in silico were then tested using conventional PCR on 10-fold serial dilutions of isolated *A. cantonensis* DNA (SYD.1) from 200 to 0.2 pg/μl. PCR amplifications were made up to 30 μl including 2 μl of template DNA, and were performed using MyTaq™ Red mix (Bioline, Australia), distilled water and primers at a concentration of 0.33 μM. Each PCR run included a no template negative control (distilled water). Cycling conditions used were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 15 s, 52°C for 15 s and 72°C for 20 s, with a final extension step at 72°C for 7 min. Selected cox1 assays capable of amplifying the highest dilution of *A. cantonensis* DNA (0.2 pg/μl) were then optimised using a temperature gradient from 52°C to 62°C, as described above, to decrease the presence of non-specific binding and primer dimers. Finally, the selected assays were tested on *A. mackerrasae* DNA isolated from an adult worm and dog DNA to determine the ability of the primer pairs to amplify both species. Two additional known-positive canine CSF samples (see Section 2.1) were included to ensure their capacity to detect *Angiostrongylus* spp. DNA in the presence of canine eosinophils. PCR products were visualised on a 2% agarose gel stained with GelRed (Biotium) and observed under UV-light.

2.4. Detection of *Angiostrongylus* spp. cox1 DNA via qPCR from the CSF of dogs with eosinophilic meningitis

The DNA from CSF samples of 61 dogs from eastern Australia with eosinophilic meningitis were screened using the selected cox1 assay via real time PCR (qPCR); of these, 50 dogs were previously confirmed PCR-positive for *Angiostrongylus* spp. DNA using a highly sensitive qPCR sensu Sears et al. (2021), while 11 were PCR-negative but antibody-positive (Lee et al., 2021). The partial cox1 PCR reaction mixtures were made up to 20 μl, including 2 μl template DNA, using Luna Universal qPCR Mastermix (New England Biolabs, Australia) and contained primers at a final concentration of 0.25 μM. The qPCR reactions were run on the CFX96 Touch Real-Time PCR Detection System (BioRad, Australia) and analysed using the corresponding CFX Maestro 1.0 software (BioRad, Australia). Cycling conditions were as follows: 95°C for 60 s, followed by 40 cycles at 95°C for 15 s and 55°C for 30 s. The cycling protocol was finished with a melt curve cycle. Each qPCR run contained reference *A. cantonensis* and *A. mackerrasae* DNA from adult nematodes as the positive controls and distilled water as a no-template negative control. Results were considered positive if the melt curve profile corresponded to that of either of the positive controls. Samples returning Ct values < 40 with expected melt curves were submitted for bidirectional sequencing at Macrogen Inc. (Seoul, Korea). DNA chromatograms were inspected for quality and ambiguity by eye and aligned to reference mtDNA genome sequences. *A. cantonensis* SYD.1 (MK570631) and *A. mackerrasae* P43/19-E (MN793157) (Cervená et al., 2019; Valentine et al., 2020). A reference alignment of all known cox1 sequences was compiled from
GenBank with the aid of tblast. Newly obtained sequences were appended to the reference alignment and percentage identity to the known cox1 haplotype was evaluated.

2.5. Detection of Angiostrongylus spp. ITS2 via qPCR from the CSF of dogs with eosinophilic meningoitis

As a nuclear marker, the second ribosomal transcribed spacer sequence ribosomal DNA (ITS2) from Angiostrongylus spp. was targeted by using a dual labelled probe AC (5'-FAM-GCT ACA TGT AAT TCG AGC ATA TGT G-BHQ-3') and two primers FP (5’-CCA GTT TTG GTG AAG AAT AA-3') and RP (5’-ACA CGA CGG TAA ACA TGA CA-3') amplifying ~140 bp product (Fang et al., 2012). The DNA from the CSF samples from the 61 dogs described in Section 2.4. were screened. The ITS2 qPCR reaction mixtures were made up to 20 μl, including 2 μl template DNA, using Luna Universal Probe qPCR Master Mix (New England Biolabs, Australia) and contained primers and probe at a final concentration of 0.25 μM and 0.2 μM, respectively. The qPCR reactions were run on the CFX96 Touch Real-Time PCR Detection System (BioRad, Australia) and analysed using the corresponding CFX Maestro 1.0 software (BioRad, Australia). Cycling conditions were as follows: 95 °C for 180 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 15 s. Each qPCR run contained reference A. cantonensis and A. mackerrasae DNA from adult nematodes as the positive controls and distilled water as the negative controls. The reactions were set up in triplicate. The data was calculated using the CFX Maestro 1.0 software (BioRad, Australia).

3. Results

3.1. In silico selection of cox1 primers to amplify A. cantonensis and A. mackerrasae

The cox1 region was selected as the mitochondrial marker for further interrogation based on the high percentage difference between the cox1 sequences (9.1%) of A. cantonensis vs A. mackerrasae, as well as the variety of cox1 primers available (n = 11) for Angiostrongylus spp. (Table 1). Five combinations of four reverse and two forward primers capable of differentiating A. cantonensis and A. mackerrasae were selected to minimise the number of primer-template mismatches (< 3) and meet the desired amplicon length (< 300 bp): Assay 1: ‘cox1F & AC1R’ primers which amplify a 259-bp region, in the position 720–979 relative to the reference cox1; Assay 2: ‘AngiCOI_forward & AC1R’ primers which amplify a 249-bp region, in the position 730–979; Assay 3: ‘AC1F & AC2R’ primers which amplify a 294-bp region, in the position 806–1100; Assay 4: ‘AC1F & AC1R’ primers which amplify a 173-bp region, in the position 806–979; Assay 5: ‘AC2F & AC2R’ primers which amplify a 149-bp region, in the position 951–1100.

3.2. Testing of different dilutions of A. cantonensis DNA to select the appropriate assay

All five cox1 assays produced a single amplicon of the expected size from A. cantonensis DNA (Fig. 1A). Assay 1 and Assay 2 amplified the highest dilution of A. cantonensis DNA (0.2 pg/μl) and were thus tested using a temperature gradient between 52 °C and 62 °C, respectively, to increase specificity and reduce primer dimer formation (Fig. 1B). Amplification of A. cantonensis DNA only occurred when the annealing temperature was ≤ 58 °C for Assay 1, while Assay 2 produced specific bands across the full temperature range. While amplification of A. mackerrasae DNA was achieved using both Assay 1 and Assay 2 primer sets, a ~700-bp nonspecific dog DNA product was produced by Assay 1 at 55 °C (Fig. 1C). The successful amplification of DNA from two selected known-positive canine CSF samples was achieved by Assay 2 but not Assay 1 at 55 °C and thus Assay 2 was chosen as the preferred assay for the amplification of Angiostrongylus DNA in canine CSF samples for further adaptation using qPCR to enable medium throughput sample processing.

3.3. Two distinct cox1 mtDNA A. cantonensis haplotypes are present in canine CSF samples from eastern Australia

Assay 2, targeting cox1 mtDNA, was used to detect Angiostrongylus spp. DNA in CSF samples of 61 dogs presenting with eosinophilic meningoitis (DOG 1-61, of which 50 PCR-positive, 11 PCR-negative but antibody-positive, using PCR (Sears et al., 2021) in Lee et al. (2021)). Assay 2 in the present study returned 12 samples (12 of 50 known PCR-positive) with C<sub>v</sub> values < 40 (C<sub>v</sub> = 28.11–36.86) and melt curves corresponding to the Angiostrongylus spp. positive controls (Table 2). Unambiguous DNA sequences with > 98% percent identity to the reference cox1 DNA sequences for A. cantonensis (MK570631) and < 95% percent identity to A. mackerrasae (MN793157) were obtained for 10 (10/12) samples. For one sample (DOG55; C<sub>v</sub> = 34.45) an incomplete DNA sequence was obtained, while the sequencing failed for another (DOG2; 36.86), likely due to low template DNA available for amplification (Table 2). In comparison with the reference cox1 haplotypes, the CSF sample from DOG53 had 100% sequence identity to cox1 of the SYD.1 reference sequence obtained from a wild rat (Rattus norvegicus) caught around the Taronga Park Zoological Gardens in Sydney 30 years ago (Cervena et al., 2019). The remaining nine samples were 100% identical.
to each other (h2) and haplotype AC13 (KU532146) from Thailand. Haplotypes SYD.1 and h2 (AC13) had three distinct nucleotide differences in the 206-bp region amplified by Assay 2 (Fig. 2A). The *A. cantonensis* cox1 positive samples screened in the present study were collected between 2010 and 2019. The lone SYD.1 haplotype was collected from a dog from Sydney in 2012, while the AC13 haplotypes detected in the remaining dogs were found in dogs from both Sydney (n = 9) and Brisbane (n = 1).

### 3.4. Verification of *A. cantonensis* identity using a nuclear ITS2 marker in canine CSF samples from eastern Australia

To verify the species identity, we included a nuclear gene, ITS2, amplified using a previously developed assay (Fang et al., 2012). The ITS2 qPCR assay amplified DNA from both *A. cantonensis* and *A. mackerrasae*. Using this assay, Angiostrongylus DNA was detected in 20/61 of the CSF samples from dogs (32.8%) presenting with eosinophilic meningitis (50 PCR-positive, 11 PCR-negative but antibody-positive using the assay by Sears et al. (2021) in Lee et al. (2021)). Unambiguous *A. cantonensis* single nucleotide polymorphisms (SNP) in the DNA sequences were obtained for 14/20 ITS2 qPCR amplicons. The key ITS2 residue between reference *A. cantonensis* and *A. mackerrasae* is at position 56 of the ITS2 amplicon, where *A. mackerrasae* has adenosine “G” while *A. mackerrasae* has guanine “A” (Fig. 2B). All 14 ITS2 sequences obtained from dog CSF samples had “G” at the 56th residue, consistent with *A. cantonensis*.

| Sample ID | Real-time PCR Assay 2 | Species (cox1) | Haplotype | ITS2 | Species (ITS2) | Ultrasensitive real-time PCR |
|-----------|-----------------------|----------------|-----------|------|----------------|-----------------------------|
| DOG37     | 28.11                  | *A. cantonensis* | AC13      | 31.36| *A. cantonensis* | 27.73                      |
| DOG57     | 31.33                  | *A. cantonensis* | AC13      | 33.01| *A. cantonensis* | 33.76                      |
| DOG58     | 32.57                  | *A. cantonensis* | AC13      | 36.92| *A. cantonensis* | 38.11                      |
| DOG23     | 33.16                  | *A. cantonensis* | AC13      | 38.21| *A. cantonensis* | 31.07                      |
| DOG8      | 33.88                  | *A. cantonensis* | AC13      | 34.57| *A. cantonensis* | 26.34                      |
| DOG49     | 34.10                  | *A. cantonensis* | AC13      | 38.71| *A. cantonensis* | fail                       |
| DOG53     | 34.20                  | *A. cantonensis* | SYD.1     | 35.55| *A. cantonensis* | 25.84                      |
| DOG36     | 34.29                  | *A. cantonensis* | AC13      | 35.70| *A. cantonensis* | 31.08                      |
| DOG55     | 34.45                  | *A. cantonensis* | fail      | 37.20| fail            | 33.08                      |
| DOG9      | 35.63                  | *A. cantonensis* | AC13      | 34.68| *A. cantonensis* | 28.29                      |
| DOG52     | 35.87                  | *A. cantonensis* | AC13      | 37.12| fail            | 29.42                      |
| DOG62     | 36.86                  | fail            | fail      | fail | fail            | 32.12                      |
| DOG11     | neg                    | *A. cantonensis* | AC13      | 33.94| *A. cantonensis* | 23.99                      |
| DOG34     | neg                    | *A. cantonensis* | AC13      | 35.62| *A. cantonensis* | 25.68                      |
| DOG50     | neg                    | *A. cantonensis* | AC13      | 35.56| *A. cantonensis* | 26.00                      |
| DOG16     | neg                    | *A. cantonensis* | AC13      | 36.09| *A. cantonensis* | 27.29                      |
| DOG12     | neg                    | *A. cantonensis* | AC13      | 37.59| *A. cantonensis* | 27.70                      |
| DOG25     | neg                    | *A. cantonensis* | AC13      | 38.54| *A. cantonensis* | 29.80                      |
| DOG43     | neg                    | *A. cantonensis* | AC13      | 37.57| fail            | 26.71                      |
| DOG42     | neg                    | *A. cantonensis* | AC13      | 38.15| fail            | 27.47                      |
| DOG61     | neg                    | *A. cantonensis* | AC13      | 38.39| fail            | 33.94                      |

* From Lee et al. (2021) using ultrasensitive real-time PCR (Sears et al., 2021).
Fig. 2. Multiple sequence alignment of the Angiostrongylus spp. DNA sequences amplified from canine CSF samples. A. A. cantonensis (SYD.1) and A. mackerrasae (ANWC:N5721) cox1 mtDNA and the novel haplotype 2 (h2) amplified using Assay 2. B. A. cantonensis (SYD.1) and A. mackerrasae (ANWC:N5721, P43/19-E) ITS2 rDNA. The ITS2 rDNA residue 56 that differentiates the two species is highlighted. The residue numbers correspond to the amplified region. Amplification primers mapped onto A. cantonensis (SYD.1) are highlighted with an arrow.

4. Discussion

To the best of our knowledge, this is the first study that unequivocally demonstrates the presence of A. cantonensis partial cox1 mtDNA and ITS2 rDNA in CSF collected antemortem from dogs with NA confirming the long-held assumption that A. cantonensis is the causal agent of canine NA. Previously, a definitive diagnosis of A. cantonensis was only possible if larvae were obtained from CSF or at necropsy and thus available for morphological analysis. While we were unable to demonstrate the presence of A. mackerrasae in canine CSF in the present study, our results have insufficient numbers to rule out the possibility that dogs may also act as accidental hosts for this species, which is important in the context of Australia where both A. cantonensis and A. mackerrasae co-exist geographically (Lee et al., 2021).

Critically, the selected mtDNA marker gene (cox1) can discriminate both A. cantonensis (MK570631) and A. mackerrasae (MN793157) species with 9% pairwise difference, making it suitable for DNA amplification and subsequent sequence comparison. Mitochondrial DNA is ideal for species differentiation in samples with low amounts of template DNA because it is present in multiple copies per individual cell (Castellani et al., 2020). For these reasons, cox1 is often used for general ‘barcoding’ of living organisms (Hebert et al., 2003). The cox1 region has the additional advantage of being one of the most commonly used markers for the identification of Angiostrongylus spp. worldwide and thus a large number of sequences are already available for comparative studies and subsequent identification of cox1 haplotypes (Monte et al., 2012; Tokiwa et al., 2012; Moreira et al., 2013; Nakaya et al., 2013; Apichat et al., 2016; Rodpai et al., 2016; Eamsobhana et al., 2017; Dusitsittipon et al., 2018; Cervenâ et al., 2019). Besides mtDNA, alternative markers include rDNA, i.e. ITS1 rDNA and ITS2 rDNA (Qvarnström et al., 2016; Lv et al., 2017).

The use of a single marker that is maternally inherited, such as those on mtDNA, may be potentially deceiving when two closely related species coexist and present the opportunity for hybridisation and introgression (Ballard & Whitlock, 2004; Harrison & Larson, 2014; Chaudhry et al., 2015). Hybridisation has been hypothesised and suggested in an experimental study between A. cantonensis and A. mackerrasae (Bhaibulya, 1974). Currently, however, no evidence suggests that hybridisation and introgression exist under field conditions in Australia, which is further supported by the results obtained in the present study where all A. cantonensis cox1 DNA-positive CSF samples had only ITS2 rDNA sequences matching A. cantonensis. Conversely, in Thailand, where A. cantonensis coexists with Angiostrongylus malayensis, a putative F1 hybrid has been identified using microsatellites analysis (Dusitsittipon et al., 2017, 2018).

Previous studies employing PCR primers targeting cox1 often used DNA isolated from Angiostrongylus nematode (adult or larvae) material and hence non-specific amplification of other parasite species or host DNA was not considered problematic (Monte et al., 2012; Tokiwa et al., 2012; Moreira et al., 2013; Okano et al., 2014; Apichat et al., 2016; Rodpai et al., 2016; Eamsobhana et al., 2017; Cervenâ et al., 2019; Valentine et al., 2020). In the present study, we used DNA isolated from canine CSF that lacked morphological evidence of the parasite, so needed to develop or adapt an assay able to amplify minimal quantities of Angiostrongylus spp. DNA (i.e. from fragments of the nematode exfoliated into CSF), while avoiding amplification of the abundant canine genomic and mitochondrial DNA (predominantly from nucleated eosinophils, the dominant cell fraction in CSF) (Lunn et al., 2012; Lee et al., 2021). This was achieved by in silico comparison of the selected primer sequences against the reference cox1 sequences of both A. mackerrasae and A. cantonensis and the adjustment of annealing temperatures to minimise amplification of host DNA. In order to facilitate this goal, we set three in silico criteria. First, primer sets needed to amplify a relatively short sequence (< 300 bp), in order to enable the amplification of potentially fragmented DNA, increase the limit of detection, and to allow adaptation of the assay for qPCR amplification (Dieffenbach et al., 1993). Secondly, the PCR amplon needed to include sufficient variation to allow discrimination between A. cantonensis and A. mackerrasae. Thirdly, a maximum of three mismatches were permitted between the primer and A. mackerrasae/A. cantonensis cox1 sequences in order to ensure stringent annealing to the target DNA. The above in silico workflow streamlined the laboratory process into empirical steps, enabling us to optimise and select existing PCR assays that met the required criteria. The final adopted assay targets a region short enough to be amplified via qPCR, enabling streamlined sample throughput, but long enough to unambiguously discriminate between A. cantonensis and A. mackerrasae mtDNA sequences.

We confirmed the presence of A. cantonensis DNA in CSF samples from 11 dogs with canine NA based on the detection of cox1 mtDNA sequences and from 14 dogs based on ITS2 rDNA sequence (Lee et al., 2021). In total, there were 16 unique dogs confirmed to possess A. cantonensis DNA in their CSF (cox1 only, ITS2 rDNA only, or both), from an original cohort of 61 dogs with NA presenting with eosinophilic meningitis, of which 50 were originally considered qPCR-positive positive for Angiostrongylus DNA using a ultrasensitive qPCR assay targeting a repetitive element conserved within both A. cantonensis and A. mackerrasae DNA (Lee et al., 2021). Although unable to differentiate Angiostrongylus spp., the ultrasensitive qPCR is considered to be 100–1,000 times more sensitive than an existing diagnostic qPCR assay targeting ITS1 rDNA (Sears et al., 2021). Our ability to amplify DNA and discriminate partial cox1 sequences in 11/50 (22%) and 14/50 (28%) ITS2 sequences from available canine CSF samples exceeded our expectations, given the limited parasite DNA on offer for detection and the known superiority of the ultrasensitive qPCR assay. There was, however, no apparent relationship between the success of our cox1 amplification and detection using the ultrasensitive qPCR for Angiostrongylus spp. DNA (see Table 2). This difference may be related to the type of DNA on offer, given that cox1 is mitochondrial DNA while the repetitive region targeted by the ultrasensitive qPCR is nuclear DNA and hence, they are potentially under different constraints (e.g. fragmentation and digestion) within CSF.
As a consequence of our successful amplification of *A. cantonensis* cox1 mtDNA in canine CSF, a new *A. cantonensis* haplotype (AC13) was discovered in Australia. Previously, it was assumed that a single parasite introduction had facilitated the establishment of the Sydney *A. cantonensis* haplotype (SYD.1) along the eastern coastline of Australia (Cervená et al., 2019). This initial incursion was always thought to have occurred in south-east Queensland rather than in Sydney, as the disease was seen in Brisbane about 20 years before it was observed in Sydney (Mackerras & Sandars, 1955; Alicata, 1991; Spratt, 2015). The results of the present study suggest that at least two, and potentially more, *A. cantonensis* introduction events have occurred in Australia. The existence of more than one haplotype outside the presumed original distribution within South East Asia is plausible as it has been previously demonstrated for specimens originating in Japan and Brazil (Monte et al., 2012; Tokiwa et al., 2012). The spread of *A. cantonensis* is facilitated by the introduction of either infected gastropod molluscs and/or rats (Rattus spp.), most likely as a result of translocation via cargo ships. Therefore, the existence of multiple cox1 haplotypes in Australia and beyond provides evidence of spread across the Pacific Ocean and South China Sea (Pien & Pien, 1999; Monte et al., 2012; Tokiwa et al., 2012, 2013; Cervená et al., 2019).

Unlike the invasive *A. cantonensis*, *A. mackerrasae* is considered a local endemic species that evolved with the Australian rat species *Rattus fuscipes* and other rodents including *Melomys cervinipes* (Bhaiubulya, 1968). The absence of *A. mackerrasae* from the sequenced results demonstrates either a sampling or detection limitation of this study, or alternatively, supports the theory that *A. mackerrasae* does not normally infect dogs. Limited information is available concerning the genetic diversity of *Angiostrongylus* spp. in Australia. Further studies on *A. cantonensis* and *A. mackerrasae* in dogs, humans and wildlife using the ultra-sensitive qPCR, alongside our cox1 assay to determine the species and haplotype from clinical samples (as in this study), are now feasible (Lee et al., 2021). Whether *A. mackerrasae* is capable of infecting other non-rodent hosts remains to be confirmed, either by direct experimental challenge, or via increased molecular surveillance. Australian wildlife species including possums, various parrot species and tawny frogmouths are considered sentinel animals for NA, with a morphologically identified specimen of *A. mackerrasae* found in a flying fox that contained first stage (L1) larvae in its faeces (Ma et al., 2013; Mackie et al., 2013). Despite these findings, the species of *Angiostrongylus* responsible for the bulk of disease in Australian wildlife has not yet been determined.

5. Conclusion

The confirmatory cox1 PCR assay adapted in our study enabled the unambiguous genetic identification of NA to the species level, thereby allowing differentiation between multiple *Angiostrongylus* spp. from antemortem canine CSF samples. While the assays were tested on canine CSF samples, they are readily applicable for use with CSF samples of non-canine origin (e.g. from human patients) or other material, including slugs and snails, as an auxiliary assay to the hyper-sensitive qPCR developed by Sears et al. (2021) or the ITS2 rDNA assay by Fang et al. (2012). Similarly, this approach can be considered for use in areas where multiple species of neurotropic *Angiostrongylus* spp. co-exist, including *A. cantonensis*, *A. malaysiensis* and *A. mackerrasae*.

**CRediT author statement**

Jeevitheswara Mallaiyaraj: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Roles/Writing - original draft; Writing - review & editing. Nichola Calvani: Formal analysis; Methodology; Validation; Writing - review & editing. Rogan Lee: Investigation; Data curation; Methodology; Resources; Writing - review & editing. Richard Malik: Investigation; Data curation; Methodology; Resources; Writing - review & editing. Jan Slapeta: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing.

**Data availability**

The nucleotide sequence data generated in this study were deposited in GenBank (NCBI) under the accession numbers MW898227–MW898236. Associated supplementary material is available at Lab-Archives (https://doi.org/10.25833/k7p1-m550).

**Declaration of competing interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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