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A Taq-Man-based multiplex quantitative PCR for the simultaneous detection and quantification of *Angiostrongylus vasorum*, *Crenosoma vulpis*, and species of respiratory capillarids in canids

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ABSTRACT

In recent years, *Angiostrongylus vasorum*, *Crenosoma vulpis*, *Eucoleus aerophilus* (syn. *Capillaria aerophila*) and *Eucoleus boehmi* (syn. *Capillaria boehmi*), commonly referred to as canine lungworms, have gained a growing interest worldwide as the result of their geographical expansion. Each of these nematode species differs considerably in its biology and pathogenicity. Despite their impact on dogs' health, these parasites are often underdiagnosed owing to diagnostic challenges. Here, we describe the development and validation of a Taq-Man-based multiplex quantitative PCR (qPCR) for the simultaneous detection of the main species of canine lungworms in faeces of infected dogs. Using 10-fold serial dilutions of synthetic gene block fragments containing individual sequence targets of each lungworm species, the analytical sensitivity of the assay ascertained was 1.84 ng/µl for *A. vasorum*, 3.08 ng/µl for *C. vulpis* and 0.79 ng/µl for *Eucoleus* spp. The sensitivity of the assays and their ability to detect mixed species infections were compared with microscopy-based techniques (faecal floatation and Baermann technique) applied to faecal samples submitted for lungworm testing through an accredited diagnostic laboratory at the Institute of Parasitology, University of Zurich, Switzerland, and from community dogs as part of a research project on canine endoparasites in Cambodia. The multiplex qPCR displayed high diagnostic sensitivity (42/46, 91.3%; 95% Confidence Interval (CI): 79.1–97.1%) and a diagnostic specificity of 100% (45/45, 95% CI: 90.6–100%), and was able to detect 42.9% additional mixed lungworm species infections compared with microscopy-based methods. Kappa statistics showed substantial agreement between the qPCRs and microscopy for mixed infections (κ = 0.72, 95% CI: 0.4–1) and *Eucoleus* spp. (κ = 0.65, 95% CI: 0.45–0.85) and almost perfect agreement for *C. vulpis* (κ = 0.85, 95% CI: 0.63–1) and *A. vasorum* (κ = 0.92, 95% CI: 0.84–1).

This multiplex qPCR enables timely, accurate, and sensitive diagnosis of canine lungworm species in faecal samples and can be used to monitor the geographical distribution and emergence of these parasitic species, globally.

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1. Introduction

The so-called canine lungworms *Angiostrongylus vasorum* (the French heartworm), *Crenosoma vulpis* (the fox bronchial worm), *Eucoleus aerophilus* (syn. *Capillaria aerophila*) (the feline and canine bronchial capillarid), *Eucoleus boehmi* (syn. *Capillaria boehmi*) (the canine nasal capillarid), and the less frequently reported metastrongyloids *Oslerus osleri* and *Filaroides hirthi*, differ in their life cycles and biology, including their predilection site within the respiratory tract and their clinicopathological impact in canid definitive hosts (DH; Deplazes et al., 2016). During the past two decades, a wealth of data on canine cardiorespiratory nematodes of dogs has aided our understanding of their biology, distribution, and pathology (Traversa et al., 2010; Di Cesare and Traversa, 2014). For instance, *C. vulpis* adults inhabit the bronchi and bronchioles of dogs (Deplazes et al., 2016), while *E. aerophilus* adults localise in

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the mucosa of the trachea, bronchi, and bronchioles (Christenson, 1938; Deplazes et al., 2016). Similarly, *E. boehmi* adults live embedded in the mucosa of nasal turbinates, frontal and paranasal sinuses (Gillis-Germitsch et al., 2020a). Conversely, *A. vasorum* has a unique localisation in the DH, with adults living in the right side of the heart, and in the pulmonary arteries where it lays eggs from which L1s hatch and pass from the blood stream to the pulmonary capillaries and trachea, and are then swallowed and passed within the faeces (Guilhon and Cens, 1973). These respiratory parasites can cause overlapping clinical signs, while differences in clinical presentation mainly reflect the final predilection site of the adult worms within the DH. *Crenosoma vulpis* is a frequent cause of chronic cough due to eosinophilic bronchitis (Shaw et al., 1996; Bihr and Conboy, 1999; Conboy, 2004), while heavy infections with *E. aerophilus* can be responsible for cough, dyspnoea, and nasal discharge owing to tracheitis, bronchitis and pneumonia (Burgess et al., 2008; Traversa et al., 2009; Companion Animal Parasite Council, 2018, available at: https://capcvet.org/guidelines/eucoleus-aerophilus/). *Eucoleus boehmi* infections can occasionally cause severe upper respiratory signs with mucopurulent nasal discharge, sneezing and reverse sneezing, owing to secondary bacterial infections (Evinger et al., 1985; Piperisova et al., 2010; Veronesi et al., 2013; Gillis-Germitsch et al., 2020a). *Angiostrongylus vasorum,* on the other hand, is capable of causing a wide range of clinical signs from respiratory distress owing to the inflammatory response to eggs and migrating larvae, to coagulopathies with local or diffuse haemorrhages that can occasionally lead to death (Chapman et al., 2004; Koch and Willesen, 2009; Di Cesare et al., 2015; Glaus et al., 2016; Sigrist et al., 2021).

In recent years, canine lungworms have gained a growing interest worldwide as the result of their geographical expansion fuelled by animal movements (Morgan and Shaw, 2010; Elsheikha et al., 2014; Gillis-Germitsch et al., 2020b).

Early detection of canine lungworms is essential to determine their changing distribution and the risk they pose to canids in both endemic and non-endemic areas, given their pathogenicity and widespread occurrence (Penagos-Tabares et al., 2018; Uribe et al., 2022).

The diagnosis of canine lungworms has traditionally relied on the identification of L1s from faeces (*C. vulpis* and *A. vasorum*) by Baerman examination, or by observation of eggs (*Eucoleus* spp.) by faecal floatation (Conboy, 2004; Traversa et al., 2010; Companion Animal Parasite Council, 2018, available at: https://capcvet.org/guidelines/eucoleus-aerophilus/). These diagnostic methods are laborious, time-consuming and, in the case of Baermann examination, require collection of fresh faecal samples, ideally analysed daily in order to overcome intermittent larval shedding (Oliveira-Júnior et al., 2006). Furthermore, the ability to differentiate L1s of closely related lungworm species and the eggs of *Eucoleus* spp. from morphologically similar trichurids (Traversa et al., 2010; Penagos-Tabares et al., 2018) is important for accurate diagnosis. In addition, co-infections with multiples species of canine lungworms can occur which can further complicate the clinical presentation and laboratory diagnosis (Bourque et al., 2002; Lempereur et al., 2016; Olivieri et al., 2017; Remesar et al., 2022).

Molecular techniques have the potential to overcome the limitations imposed by traditional coproscopic methods, given their ability to detect low levels of pathogen DNA whilst simultaneously accurately identifying different species. However, to date only a few molecular assays are available for the diagnosis of single species of lungworm infections including a probe-based real-time PCR targeting the second ribosomal transcribed spacer (ITS-2) rRNA gene of *C. vulpis* (Schug et al., 2018); a SYBR green real-time PCR targeting the ITS-2 rRNA gene of *A. vasorum* (Jefferies et al., 2009), a PCR-RFLP targeting either the ITS-2 rRNA gene or the mitochondrial DNA (mtDNA) cytochrome oxidase c subunit 1 (*cox*1) regions of *A. vasorum* (Jefferies et al., 2009; Caldeira et al., 2003) and PCRs targeting the *cox*1 gene of *Eucoleus* spp. (Di Cesare et al., 2012, 2015; Guardone et al., 2013). Taq-Man-based assays have been proven to be superior to conventional PCR (cPCR) for the detection of parasites present in the faeces (Paula et al., 2015; Hii et al., 2018; Massetti et al., 2020), especially in detecting mixed species infections, whilst also representing an ideal high-throughput diagnostic method suitable for large epidemiological studies.

In this study, we developed and optimised a novel Taq-Man-based multiplex quantitative PCR (qPCR) able to simultaneously screen for the main species of canine lungworms. Diagnostic parameters of the multiplex qPCR assays were validated and compared with the Baermann technique and faecal floatation by using faecal samples submitted for lungworm testing through an accredited diagnostic university laboratory in Switzerland, and samples collected from community dogs as part of a research project on canine endoparasites in Cambodia.

2. Materials and methods

2.1. Parasite materials

Dog faeces positive for eggs of *Eucoleus* spp. and for L1s of *C. vulpis,* and *A. vasorum* were obtained from samples submitted to the diagnostic unit of the Institute of Parasitology, University of Zurich, Switzerland. The positive samples were suspended in 0.5 ml of ethanol and sent to the Parasitology division of the University of Melbourne, Australia. Genomic DNA was extracted from each sample using the Fecal Isolate II DNA extraction kit (Bioline, London, United Kingdom) as per the manufacturer's instructions. All applicable international and institutional guidelines for the care and use of animals were followed: material from naturally infected animals were obtained in the frame of diagnostic analyses.

Faecal samples from dogs in Cambodia were part of a cross-sectional study (Zendejas-Heredia et al., 2023) and taken directly from the rectal ampulla using a glove and lubricant, ensuring the animal was subjected to minimal stress and discomfort. Faecal samples were immediately homogenised in 5% w/v potassium dichromate and kept on ice until refrigerated at 4 °C. Samples were then air-transported to the Faculty of Science, University of Melbourne on ice and kept refrigerated at 4 °C until subjected to genomic DNA extraction as reported in Zendejas-Heredia et al., 2023. Ethical committee approval was obtained from the University of Melbourne Animal Ethics Committee (approval number 1814620.1).

2.2. Singleplex and multiplex qPCR assays

The multiplex qPCR assay was designed to detect the ITS-2 rRNA region of *A. vasorum* and *C. vulpis,* and the 18S rRNA of respiratory *Eucoleus* spp. Reference nucleotide sequences for the ITS-2 rRNA of *A. vasorum* (Reference GenBank accession number MN178647, MK675820, KF270683, GU045376, EU915248, EU915248, EU627598), *C. vulpis* (Reference GenBank accession number KF836608, MT808324, MT808325, MH78005), and the 18S rRNA of the respiratory species of *Eucoleus* (Reference GenBank accession number KJ579703, KJ579702, KJ579696, KJ579693, KJ579692, KJ579669, KJ579676, KJ579683) were obtained from NCBI (https://www.ncbi.nlm.nih.gov) and aligned with GenBank sequences sourced from the available range of geographical isolates for each lungworm species, as well as other closely related canine helminths, using Geneious Prime 2019.1.1 (https://www.geneious.com). Primers and probes targeting conserved regions of the ITS-2 and 18S regions were designed with Geneious

Prime and checked for suitability using Oligo Analyzer (Integrated DNA technologies, https://sg.idtdna.com/calc/analyzer), and their genus- or species-based specificity assessed using NCBI Blast (https://www.ncbi.nlm.nih.gov/blast). The primers were manufactured by Integrated DNA Technologies, USA. The probes were designed and manufactured using locked nucleic acid (LNA) technology (Integrated DNA Technologies, USA) to increase probe specificity. A four-channel Magnetic Induction Cycler (BioMolecular Systems, Sydney, Australia) was used for the amplification, detection and data analysis (micPCR software).

The multiplex qPCR used three sets of primers; a previously published set of primers to amplify a 121 bp region of the ITS-2 rRNA gene of *A. vasorum* (Jefferies et al., 2011); a primer pair designed to amplify a 213 bp region of the ITS-2 rRNA gene of *C. vulpis* (CVULPISits2F and CVULPISits2R), and a primer pair targeting a 153 bp region of the 18S rRNA gene of *Eucoleus* spp. (EUCF and EUCR). Mammalian primers (Mam F and Mam R) and probe (MAM PROBE) were used in each reaction according to Massetti et al. (2020) as DNA extraction and amplification controls.

The qPCR assays were conducted in 20 µL reactions containing 10 µL of GoTaq Probe qPCR Mastermix (Promega, Madison, WI, USA), and 2 µL of template DNA. Nuclease-free water was added to reach the final reaction volume. The fluorescent threshold was set to 5% for all targets. The cycling conditions were identical for both reactions: 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. gBlocks Gene Fragments (IDT Technologies, Skokie, Illinois, USA) were used as positive controls and nuclease-free water was used as the negative control in all runs.

Optimization of each singleplex and multiplex assay was carried out using synthetic double-stranded DNA fragments (gBlocks Gene Fragments) containing individual sequence targets of each species. Progressive concentrations of primer pairs and probes were tested to select the concentrations that guaranteed the highest efficiency and the greatest sensitivity.

The efficiency and the intra-assay reproducibility of multiplex reactions were directly compared with each singleplex reaction by plotting the cycle threshold (Ct) versus 10-fold serial dilutions of target gBlock Gene Fragments of known DNA concentration. The correlation coefficients (R^2) of the standard curves were produced automatically by micPCR software (BioMolecular Systems, Sydney, Australia).

2.3. Sensitivity and specificity of the qPCRs

The analytical sensitivity (i.e. the ability of an assay to detect the lowest concentrations of a given target substance or organism in a biological sample (Saah, 1997)) of the qPCRs was assessed using 10-fold serial dilutions of target gBlock Gene Fragments of known DNA concentration (i.e., 1.84 ng/µl for *A. vasorum*, 3.08 ng/µl for *C. vulpis,* 0.79 ng/µl for *Eucoleus* spp.).

To investigate the analytical specificity of the assay (i.e. the ability of an assay to exclusively identify a particular target substance or organism rather than similar but different substances or organisms (Saah, 1997)), this newly developed qPCR protocol for canine lungworms was tested against genomic DNA extracted from the eggs or L1s of each of the species of canine lungworms targeted by the qPCR and genomic DNA of the most common parasites infecting dogs, namely *Toxocara canis, Toxascaris leonina, Taenia* spp., *Spirometra erinaceieuropaei, Strongyloides* spp., *Giardia duodenalis, Trichuris vulpis*, the canine hookworms *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma braziliense* and *Uncinaria stenocephala*, and nematodes phylogenetically related to canine lungworms i.e. *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis.*

A total of 157 faecal samples were utilised to assess the diagnostic parameters of the qPCR, consisting of canine faecal samples submitted for lungworm testing through an accredited diagnostic laboratory at the Institute of Parasitology, University of Zurich, Zurich, Switzerland (n = 89), and from community dogs as part of a research project on canine endoparasites in Cambodia (n = 68). For the samples from Switzerland, eggs were isolated with the combined sedimentation-floatation method using zinc chloride saline solution (SpGr 1.45) and L1s of lungworms with the Baermann technique, and both were differentiated using morphological keys (Deplazes et al., 2016). Faecal samples of dogs from Cambodia were subjected to zinc chloride saline solution (SpGr 1.3) or sodium nitrate faecal floatation to screen for helminth eggs, and identified using morphological keys (Deplazes et al., 2016). Comparison between microscopy (i.e. Baermann technique for *C. vulpis* and *A. vasorum* and faecal floatation for *Eucoleus* spp.) and qPCR was performed, relying on Kappa statistics (Watson and Petrie, 2010), considering all faecal samples from Cambodia and Switzerland. Comparison between Baermann technique and qPCR was performed using the Swiss samples. Agreement between assays was considered poor if the coefficient (k) < 0.00, slight if $0.00 \leq k \leq 0.20$, fair if $0.21 \leq k \leq 0.40$; moderate if $0.41 \leq k \leq 0.60$, substantial if $0.61 \leq k \leq 0.80$ and almost perfect if $k > 0.80$.

The diagnostic sensitivity was assessed on a subset of 46 faecal samples deemed positive for canine lungworms by faecal floatation (*Eucoleus* spp.) or Baermann technique (*A. vasorum* and *C. vulpis*), and calculated as the number of samples correctly identified as positive by the qPCR (true positives) divided by the total number of microscopy positive samples (true positives and false negatives).

Assessment of the overall diagnostic specificity (i.e., the ability of an assay to correctly classify an individual as negative if the individual is truly negative) was calculated for 45 faecal samples deemed negative by both faecal floatation and Baermann technique, and calculated as the number of samples correctly identified as negative by the qPCR (true negative) divided by the total number of microscopy negative samples (true negatives and false positives). The 95% confidence intervals (95% CI) were calculated using the modified Wald method (Agresti and Coull, 1998).

2.4. Generation of the formula to convert Cycle quantification (Cq) values to eggs per gram (epg) (Eucoleus spp.) and larvae per gram (lpg; A. vasorum, C. vulpis)

Six serial dilutions were produced using known quantities (100, 50, 20, 10, 5, 1) of *Eucoleus* spp. eggs and L1s of *C. vulpis* and *A. vasorum* isolated from selected faecal samples and seeded in 200 mg of faeces in triplicate. Genomic DNA was extracted from each faecal sample spiked with known number of eggs/L1s using the Faecal Isolate II DNA extraction kit (Bioline) as per the manufacturer's instructions. The final elution volume was 30 µl. Two microliters of DNA were added to each multiplex lungworm qPCR, and non-template controls included in each run. Egg and larval counts expressed in eggs per gram (epg) and L1s per gram (lpg) were calculated by multiplying the absolute egg count by a factor of 5, as 200 mg of faecal sample were subjected to DNA extraction for the current study.

3. Results

3.1. Canine lungworms multiplex qPCR optimization

The optimised primer and probe concentrations are listed in Table 1. qPCR efficiencies ranged from 90% to 96% with an R^2 of 0.999 and Slope from −3.44 to −3.58 (Fig. 1).

Table 1
Oligonucleotide primers and probes for quantitative PCR (qPCR) assays for the detection of *Angiostrongylus vasorum, Eucoleus* spp., and *Crenosoma vulpis.*

Target	Primers and probes	Sequence (5′–3′)	Gene target	Size(bp)	Conc. nm	Source
A. vasorum	AVASORUMF	ACGCATGATGAAAGAATGCTGG	ITS-2 rRNA	121	400	Jefferies et al., 2011
	AVASORUMR	GACGACGACGACAACCACT			400	Jefferies et al., 2011
	AVASORUM Probe	56-FAM/CA TGT TT + G GAC TA + T TTG T + CA AAC GG/3IABkFQ/			300	This study
Eucoleus spp.	EUCF	GAGCTAATACACGCCTTGATG	18 s rRNA	153	300	This study
	EUCR	GCAAGTCGTTTCAAAGATTCGTC			300	This study
	EUC PROBE	5HEX/CA ATT TTG + ATC GGC TCG C + TC GC/3IABkFQ/			60	This study
C. vulpis	CVULPISits2F	GATAGTGTAGATCATCATTGCTAC	ITS-2 rRNA	213	200	This study
	CVULPISits2R	CGGGTAATCACGTCTGAGCT			200	This study
	CVULPIS Probe	/5Cy5/CG TCG + AGG TGG TTT + TCA + AAC G/3IAbRQSp/			300	This study

Conc., Concentration.

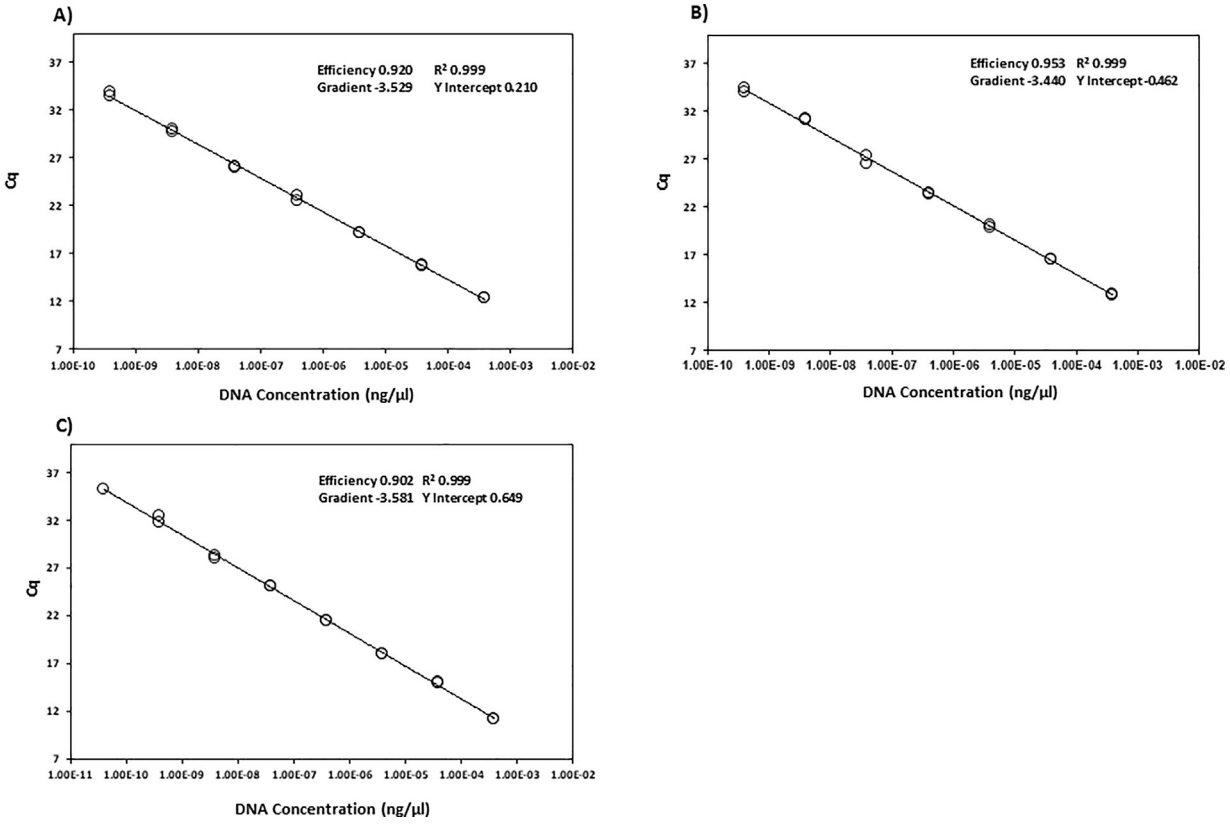


Fig. 1. Standard curves generated from 10-fold serial dilutions of target gBlock Gene Fragments of the lungworms *Crenosoma vulpis* (A), *Angiostrongylus vasorum* (B), *Eucoleus* spp. (C). Cq, Cycle quantification.

3.2. Sensitivity and specificity of the qPCRs

Mean Cq values associated with minimum detection limits were 37.16 (±0.24 S.D.) for *C. vulpis*, 36.89 (±0.368 S.D.) for *A. vasorum,* and 37.02 (±0.4 S.D.) for *Eucoleus* spp., which corresponded to an analytical sensitivity of 6.16×10^{-3} fg of target gBlock Gene DNA for *C. vulpis*, 3.7×10^{-3} fg of target gBlock Gene DNA for *A. vasorum*, and 1.76×10^{-3} fg for *Eucoleus* spp. (Fig. 2). The qPCR was able to detect up to five lpg of *A. vasorum* and *C. vulpis,* and five epg of *Eucoleus* spp., which corresponded to the smallest quantity of larvae and eggs (i.e. one) present in 200 mg of faces used for DNA extraction (See Fig. 3).

The qPCR correctly identified the genomic DNA extracted from the L1s *(A. vasorum* and *C. vulpis*) or eggs (*Eucoleus* spp.), without cross-reactions between the targeted species. The multiplex qPCR assays did not cross-react with genomic DNA of the non-target parasites, thereby displaying high analytical specificity.

The multiplex qPCR displayed an overall diagnostic sensitivity of 91.3% (42/46; 95% CI: 79.1–97.1%) using microscopy-based techniques as the gold standard. The diagnostic specificity calculated on the samples confirmed negative to both Baermann technique and faecal floatation was assessed as 100% (45/45, 95% CI: 90.6–100%).

3.3. Performance of the multiplex qPCRs for the detection of DNA of canine lungworm species in faecal samples

The results of the multiplex quantitative qPCR compared with microscopy are summarised in Table 2. Kappa statistics showed substantial agreement between the qPCRs and microscopy for mixed infections (κ = 0.72, 95% CI: 0.4–1) and *Eucoleus* spp. (κ = 0.65, 95% CI: 0.45–0.85). Almost perfect agreement was shown for *C. vulpis* (κ = 0.85, 95% CI: 0.63–1) and *A. vasorum* (κ = 0.92, 95% CI: 0.84–1). The qPCR was able to detect an additional three

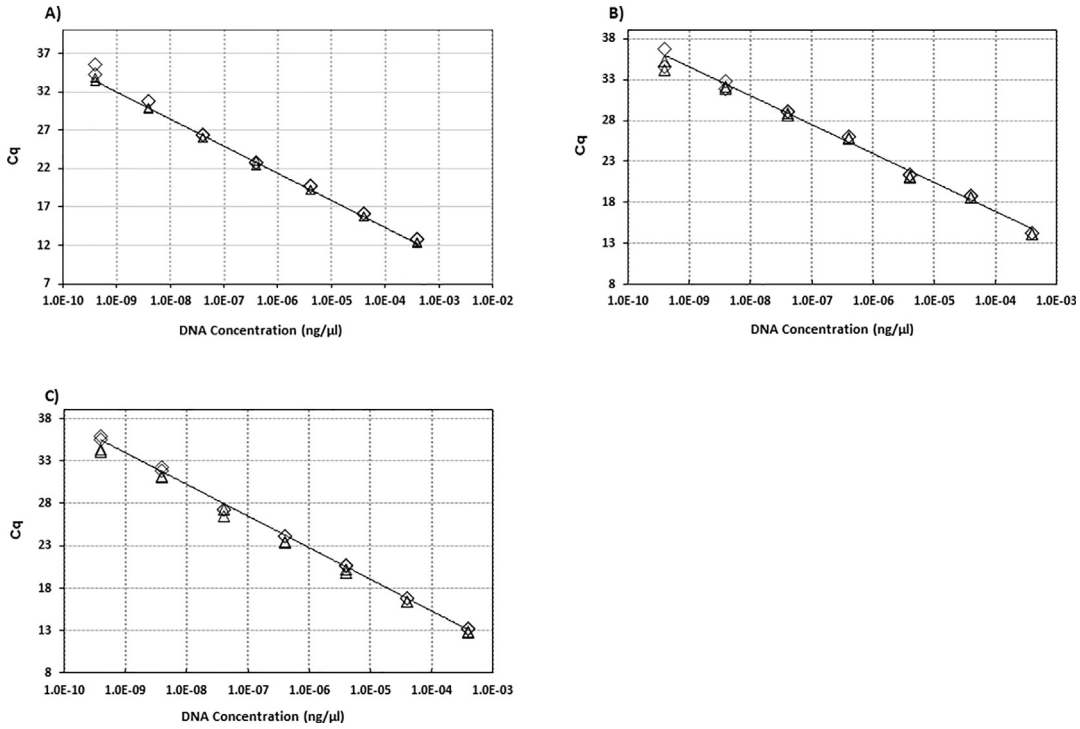


Fig. 2. Singleplex and multiplex quantitative PCR efficiencies. Optimisation and comparison of the sensitivity and efficiency of each singleplex (triangles) and multiplex qPCRs (squares) using gBlock Gene Fragments controls for *Crenosoma vulpis* (A), *Angiostrongylus vasorum* (B), and *Eucoleus* spp. (C). Cq, Cycle quantification.

Table 2
Multiplex quantitative PCR (qPCR) and microscopy (Baermann and faecal floatation) agreement statistics. The comparison between the Baermann technique and qPCR was performed on a subset of 89 faecal samples. The comparison between faecal floatation and qPCR was performed on a total of 157 faecal samples.

	qPCRs	Microscopy		Total Agreement %	Kappa (95% CI)
		POS	NEG		
C. vulpis	POS	6	0	98%	0.85 (0.63–1)
	NEG	2	81		
A. vasorum	POS	28	3	97%	0.92 (0.84–1)
	NEG	0	58		
Eucoleus spp.	POS	12	8	93%	0.65 (0.45–0.85)
	NEG	3	134		
Mixed infections	POSNEG	40	3150	98%	0.72 (0.4–1)

POS, positive; NEG, negative; 95% CI, 95% confidence interval.
K agreement level: $k < 0.00$ poor; $0.00 \leq k \leq 0.20$, slight; $0.21 \leq k \leq 0.40$, fair; $0.41 \leq k \leq 0.60$, moderate; $0.61 \leq k \leq 0.80$; substantial; $k > 0.80$ almost perfect.

A. vasorum (9.7%), eight *Eucoleus* spp. (34.7%), and three *Eucoleus* spp. and *A. vasorum* mixed infections (42.9%) compared with microscopy.

3.4. Formulas to convert Cq values to epg or lpg of faeces

The Cq values plotted against known seeded quantities of purified eggs of *Eucoleus* spp. and L1s of *A. vasorum* and *C. vulpis,* and the conversion formulas for Cq to absolute egg/larval counts (2 µL of DNA/qPCR assay) are shown in Fig. 3. There was a strong linear correlation between Cq values of *Eucoleus* spp., *A. vasorum*, *C. vulpis* and the $\log_{10}$ of the absolute egg/larval counts (R^2 between 0.94 and 0.98). The conversion formula for Cq to egg intensities (epg) and larval intensities (lpg) was $10^{(Cq-30.296)/-3.244}$ for *A. vasorum*; $10^{(Cq-32.989)/-3.5558}$ for *C. vulpis,* and $10^{(Cq-35.198)/-2.953}$ for *Eucoleus* spp.

4. Discussion

Here, we developed and validated a Taq-Man-based multiplex qPCR for the simultaneous detection of the main species of canine lungworms from faecal samples. Such a diagnostic tool will support clinicians and researchers to accurately diagnose the main species of canine lungworms in a timely manner and assist large epidemiological field studies to monitor emergence and spread of these pathogens globally.

In recent decades, an increasing number of reports of canine lungworms, especially *A. vasorum,* has been documented worldwide, highlighting the fast-paced geographical expansion of these parasites (Duarte et al., 2007; Bridger and Whitney, 2009; Gerrikagoitia et al., 2010; Conboy, 2011; Al-Sabi et al., 2014; Elsheikha et al., 2014; Santoro et al., 2015; Alho et al., 2018; Deak et al., 2019; Gillis-Germitsch et al., 2020b). Knowledge on the epidemiology of canine lungworms is critical for clinicians,

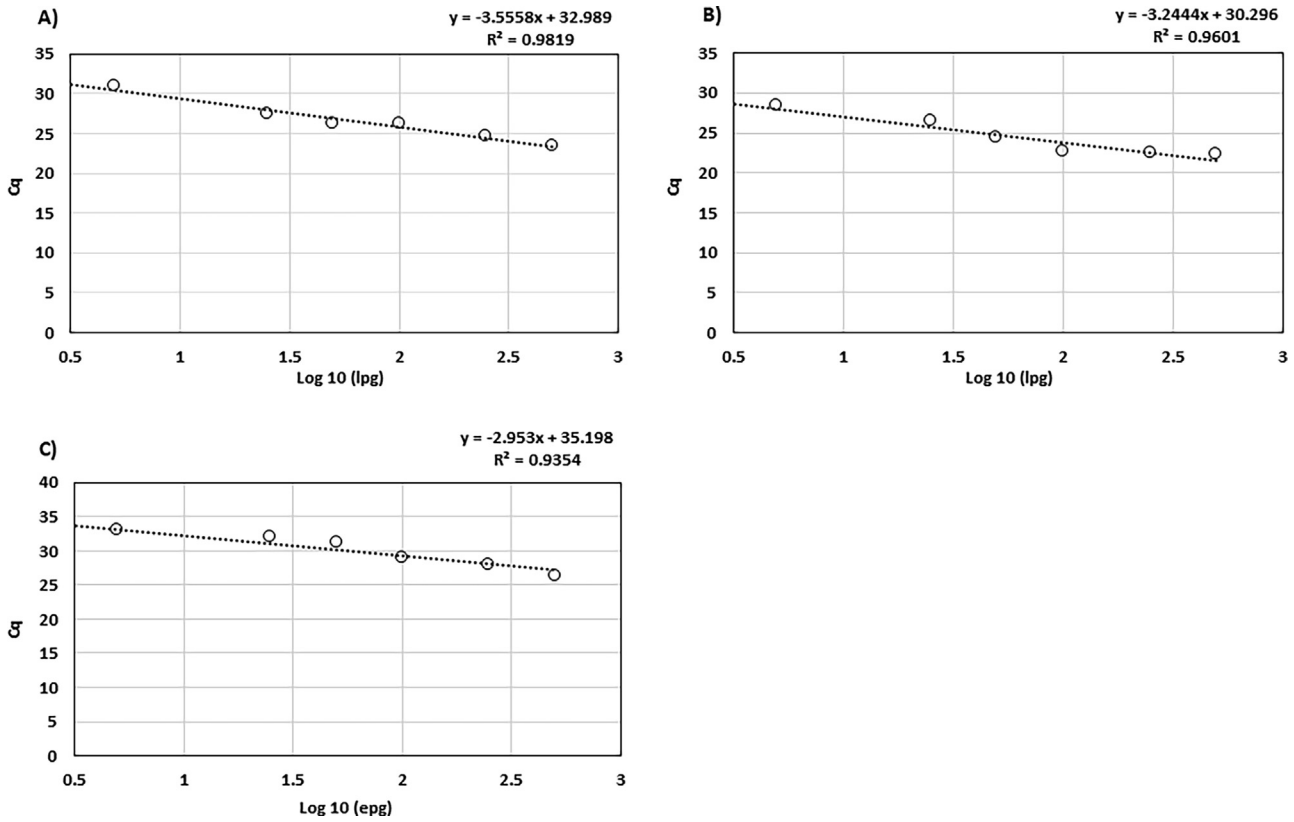


Fig. 3. Cycle quantification (Cq) values plotted against known quantities of *Crenosoma vulpis* (A), and *Angiostrongylus vasorum* (B) L1s, and seeded eggs of *Eucoleus* spp. (C). An excellent correlation was observed between seeded average egg/larval counts and average Cycle quantification (Cq) measured by the multiplex quantitative PCR. epg, eggs per gram; lpg, larvae per gram.

given the susceptibility of dogs to infections and the potentially fatal disease outcome associated with *A. vasorum*. Veterinarians and owners might become aware of the infection only when severe clinical signs occur, e.g. acute cardiorespiratory or neurological signs and bleeding associated with *A. vasorum* infections (Chapman et al., 2004; Glaus et al., 2016; Sigrist et al., 2017). As such, the prompt, sensitive and specific diagnosis of these pathogens acquires pivotal importance.

However, diagnoses of canine lungworm infections are challenging due to the often dominating non-specific clinical signs, the initially frequent sub-clinical course of the disease, and the partially low sensitivity associated with coprological techniques (Traversa and Guglielmini, 2008; Traversa et al., 2010; Schnyder et al., 2011a, 2011b; Canonne et al., 2018).

Our newly developed multiplex qPCR meets such requirements, whilst also providing relatively rapid diagnosis compared with the time-consuming and less sensitive traditional techniques such as the Baermann method, faecal floatation, and singleplex PCRs. For instance, the Baermann technique, the most commonly used diagnostic for larval identification, requires from 12 to 24 h, and faeces as fresh as possible, to achieve diagnosis (Oehm and Schnyder, 2022). In contrast, qPCR does not require fresh faecal material, hence representing an ideal diagnostic tool for large epidemiological studies, comparable with serological screening. In fact, serological tests for detection of circulating antigens and of antibodies against *A. vasorum* have been repeatedly used for large dog population studies (Schnyder et al., 2013; Globokar et al., 2021), however, there are no serological tests available for the detection of other canine lungworms.

Therefore, another advantage of the qPCR over conventional techniques is the possibility to identify all of the main species of canine lungworms in a single assay in as little as 200 mg of faecal material, compared with the recommended 4–10 g required for the Baermann technique (*A. vasorum* and *C. vulpis*), and in addition, 4–5 g for faecal floatation (*Eucoleus* spp.) (Tropical Council for Companion Animal Parasites, 2019, available at: https://www.troccap.com/2017press/wp-content/uploads/2019/05/TroCCAP_Canine_Endo_Guidelines_English_Ver2.pdf). Furthermore, faecal samples can be easily collected by lay persons, the procedure being less invasive than blood collection, and when destined for qPCR faeces can be stored and transported at room temperature to laboratories with qPCR capabilities without the need of special equipment such as the Baermann apparatus or trained technicians for parasites identification, making it an ideal tool for large-scale surveys.

Singleplex qPCR assays for the detection of canine lungworms have already been developed for *A. vasorum* and *C. vulpis* (Jefferies et al., 2009, 2011; Schug et al., 2018) and for the delineation of species in the family Trichuridae (Guardone et al., 2013). However, none was able to detect all lungworms simultaneously. Furthermore, compared with previously developed qPCR assays targeting canine lungworms, we improved the sensitivity and specificity of this assay by using LNA technology, i.e. modified nucleotide bases which reduce background noise, and greatly increase hybridization specificity, allowing a better mismatch discrimination in single nucleotide polymorphism (SNP) genotyping assays.

For instance, our qPCR demonstrated an overall diagnostic sensitivity of 91.3% (42/46; 95% CI: 79.1–97.1%) and a diagnostic specificity of 100% (45/45, 95% CI: 90.6–100%) using microscopy as the gold standard. The diagnostic sensitivity of this assay found in this study was superior to the Angio Detect™ Test (IDEXX Laboratories,

Westbrook, Maine, USA) rapid assay for the detection *of A. vasorum* antigen from blood (84.9%; Schnyder et al., 2014), and similar to an ELISA also detecting circulating antigens of *A. vasorum* from serum (94.9%) (Schnyder et al., 2011a, 2011b, 2014). Kappa statistics showed substantial to almost perfect agreement between microscopy and the qPCR, confirming the reliability of this method. However, coproscopy relies on the excretion of parasite sexual stages such as eggs and L1s: their excretion may be intermittent or suppressed. Also, coprophagia may interfere with the identification of true infected animals. Considering this, *A. vasorum* serology, compared with copromicroscopic methods relying on the detection of L1s, was shown to overcome the effects of intermittent larval shedding, leading to better sensitivity (Schnyder et al., 2015).

Similar to previous studies, one of the main advantages of the qPCR is its capability of detecting additional mixed species infections (Hii et al., 2018; Massetti et al., 2020). The qPCR herein developed was able to detect additional mixed lungworm species infections (42.9%) compared with microscopy. A small number of samples were qPCR-negative and microscopy-positive, however, in the case of low larval output or low egg-shedding infections, the Baermann technique and faecal floatation might prove more sensitive, owing to the greater possibility of eggs/L1s being present in higher amounts of faeces compared with the 200 mg of faecal material used for DNA extraction.

Recently, it has been shown that the level of lpgs is related to worm burdens for *A. vasorum* (Oehm and Schnyder, 2022). Here, we identified a strong linear correlation between Cq values of all investigated lungworms and the absolute egg/larval counts. Therefore, this qPCR has the potential to aid veterinarians to assess the severity of clinical cases by predicting the lpg and correlated worm burdens associated with each Cq. Nevertheless, further investigations on the correlation between clinical signs and worm burden in adult dogs, using Ct conversion formulas, are required.

This assay has proven specific as it detected all the main species of canine lungworms without cross-reacting when tested against a range of intestinal parasites of dogs, including the congeners *A. cantonensis* and *A. costaricensis*. However, the primers designed to amplify the ITS-2 rRNA of *C. vulpis* matched with a single sequence previously identified as *Cercopithifilaria* sp. (JF501396.1). This sequence was misclassified as *Cercopithifilaria,* given the phylogenetic distance between the latter and *C. vulpis*, and the high nucleotide identity (100%) of this sequence (i.e. JF501396.1) with *Crenosoma* ITS-2 rRNA sequences available from NCBI (OM480716.1; OM914984.1; KF836608.1; MT808324.1; OM480715.1).

A limitation of this qPCR is its inability to differentiate *E. aerophilus* from *E. boehmi*. However, a semi-nested PCR for the specific identification of *E. boehmi* is available and might be performed on the respiratory capillarid qPCR-positive samples to distinguish between the species (Di Cesare et al., 2015), when discernment is needed.

Furthermore, in order to assess the diagnostic parameters of the newly developed qPCR we performed microscopy (considered in this study as a gold standard) on samples from Cambodia and Switzerland. Owing to the challenge of diagnosing canine lungworms and to the low egg/larval output, it is plausible that microscopy might have failed in detecting a number of positives. For instance, eight samples from Cambodia not subjected to the Baermann technique but negative on faecal floatation for respiratory capillarids yielded positive for *Eucoleus* spp. by qPCR.

Accordingly, owing to the unavailability of fresh faecal material, the diagnostic specificity was calculated only on the subset of samples from Switzerland, previously confirmed negative for canine lungworms by both the Baermann method and faecal floatation.

Overall, the ability of this qPCR assay to simultaneously detect the main species of canine lungworms from faeces with high sensitivity and specificity makes it a useful tool for researchers to conduct worldwide epidemiological studies and track the geographical spread of these parasites. Molecular assays have proven fundamental to investigation of the epidemiology of canine lungworms in both the intermediate and DH in endemic areas of northern Europe (Aziz et al., 2016; Schug et al., 2018). The employment of molecular assays also allowed identification of the zoonotic rat lungworm *A. cantonensis* from non-endemic areas of Europe (Foronda et al., 2010; Paredes-Esquivel et al., 2019). Similarly, this newly developed qPCR has the potential to assist the high-throughput monitoring of canine lungworms worldwide in both endemic and non-endemic areas. In addition, this qPCR will aid researchers and veterinarians in making precise diagnoses of canine lungworm infections, overcoming the limitations imposed by microscopy-based analysis, including the need for expert knowledge to distinguish *A. vasorum* and *C. vulpis* L1s and other interfering larvae, and eggs of *Eucoleus* spp. from those of some other Trichuridae (Bihr and Conboy, 1999; Conboy, 2009; Traversa et al., 2010; Penagos-Tabares et al., 2018). Veterinary laboratories with qPCR facilities adopting this tool will enable prompt detection of canine lungworm infections and consequent treatment of infected animals, possibly before the emergence of severe and irreversible clinical signs. The employment of this qPCR may also enable an accurate assessment of the evolving epidemiological scenarios of canine lungworms in endemic and non-endemic areas, on a large scale. This will aid in identifying key drivers behind the spread of canine lungworms worldwide and support local animal health institutions in tackling their expansion through adoption of appropriate preventive measures.

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