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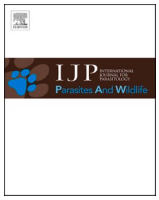

Validation of a species-specific probe-based qPCR for detection of *Setaria yehi* (Filarioidea: Onchocercidae) in Alaskan moose (*Alces alces gigas*)

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ABSTRACT

Northern ungulates contend with *Setaria yehi* and *Rumenfilaria andersoni*, filarioid nematodes that are transmitted by ectoparasitic blood-feeding arthropods, which can result in animal and population level impacts. *Setaria yehi* microfilariae can be detected in fresh blood samples using a modified Knott's test, or by postmortem detection by genetic sampling or through the retrieval of adult specimens in the peritoneal cavity. In this study we validated a novel qPCR for detection of *S. yehi* DNA in blood samples of moose (*Alces alces*). Additionally, we compared quantitative values from modified Knott's test to detect both *S. yehi* and *R. andersoni* from both fresh and frozen blood samples. Species-specific primers targeting a 121-base pair fragment of the cytochrome oxidase c subunit 1 (*cox1*) of *S. yehi*, and a species-specific probe were designed. The qPCR had a detection threshold of 0.157 pg/μL of parasite DNA. We collected 166 blood samples from wild moose captured on the Kenai Peninsula, Alaska from 2019 to 2022. Matching blood aliquots were tested by modified Knott's test and subjected to DNA extraction for subsequent qPCR. Quantitatively, blood samples had an average *S. yehi* microfilaremia (mf) of 472.2 mf/mL (0–14,490 mf/mL) and *R. andersoni* of 72.9 mf/mL (0.0–5071.5 mf/mL). Qualitatively, 32.53% ($n = 54$) of samples tested positive for *S. yehi* in each of the tests, and 37.35% ($n = 62$) when both tests were combined, with very good agreement between the results from Knott's test and qPCR (*kappa* = 0.90). The validation of the qPCR test for *S. yehi* allows for faster, less labor-intensive diagnosis and epidemiological surveillance of this emerging parasite in moose and other cervid hosts.

1. Introduction

Moose (*Alces alces*), a circumpolar northern ungulate inhabiting temperate, boreal, and arctic regions, are exposed to a wide variety of parasites across their range (Hundertmark, 2016). Both ectoparasites and endoparasites affect moose across its range in North America, with the level of impact varying from relatively minor to morbidity and mortality (Pybus et al., 2001; Kutz et al., 2012). Ectoparasite infestations are quickly detected as visual impacts are noticeable to both biologists and the public. Moose flies (*Haematobosca alcis*) inflict open sores on the hind legs of moose, while winter ticks (*Dermacentor albipictus*) result in hair loss during winter (Benedict et al., 2023; Lankester and Samuel, 2007; Lankester and Sein, 1986; McLaughlin and Addison, 1986; Murie, 1934). Infections by some helminth endoparasites (e.g., giant liver fluke (*Fascioloides magna*); meningeal worm (*Parelaphostrongylus tenuis*) in moose are detected during post-mortem inspections (Grunenwald et al., 2018; Haake et al., 2024; Henningsen et al., 2012; LeVan et al., 2013; Madden et al., 1991; Maskey, 2011; Pybus et al., 2001; Shury et al., 2019; Whitlaw and Lankester, 1994; Worley and Anderson, 1972; Wünschmann et al., 2015); however, infections by some filarioid nematodes of the genera *Setaria*, *Rumenfilaria*, *Onchocerca*, and *Elaeophora* can be detected from live moose using blood samples or skin biopsies (Anders et al., 2024; Benedict et al., 2023; Grunenwald et al., 2016; Kutz et al., 2012; Lankester and Snider, 1982; Pledger, 1978; Samuel et al., 1976; Verocai et al., 2012; Williams and Babero, 1958) These filarioid nematodes (Filarioidea; Onchocercidae) are of particular relevance as they are transmitted by ectoparasitic blood-feeding arthropods (Anderson, 2000), and infections have been documented to have both individual animal and population level impacts on northern ungulates (Kutz et al., 2012; Laaksonen et al., 2009, 2010).

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Setaria spp. are one of the most common mosquito-borne, filarioid nematodes that infect moose and other wild and domestic ungulates globally (Anderson, 2000; Desset, 1966). *Setaria yehi* Desset (1966), or the "abdominal worm", infects various wild northern cervids, including moose, caribou (*Rangifer tarandus*), mule deer (*Odocoileus hemionus*) and white-tailed deer (*Odocoileus virginianus*) (Becklund and Walker, 1969; Fruetel and Lankester, 1989; Grunenwald et al., 2016; Kutz et al., 2012; Weinmann et al., 1973; Yeh, 1959).

Setaria species require a definitive mammal host that acquires the *Setaria* third-stage larvae (L3) upon the bloodmeal from an infected mosquito (Diptera; Culicidae), the intermediate host. The *Setaria* L3 will then develop and migrate to the peritoneal cavity of the mammalian host, where it will mature and sexually reproduce. Adult female *Setaria* will then release microfilariae, which then enter circulation of the blood stream of the mammalian host and will be ingested by another mosquito. Within the mosquito, the *Setaria* microfilariae will develop through L3 infective stage, when it will migrate to the salivary glands of the mosquito (Anderson, 2000). Various species of *Setaria,* including *S. yehi,* have been reported to cause prenatal transmission when the migrating larvae reach the fetal tissues of a pregnant mammal (Gomez-Puerta and Mayor, 2017; Weinmann et al., 1973). Thus, the neonate may be born infected with *Setaria*, and the nematodes are well advanced in their development, which can establish a patent infection in the calf within a few months.

Other filarioid nematodes reported from moose in North America are *Rumenfilaria andersoni* Lankester and Snider (1982); *Elaeophora schneideri* Wehr and Dikmans, 1935; and at least two distinct species of *Onchocerca*, comprised within the *Onchocerca cervipedis* species complex (Benedict et al., 2023; Kulpa et al., 2021; Lefoulon et al., 2017; Verocai et al., 2012). *Rumenfilaria andersoni* is a filarioid nematode whose adults are found in the lymphatic vessels of the rumen of moose and white-tailed deer in North America (Grunenwald et al., 2016; Lankester and Sein, 1986), with evidence of its introduction and emergence in reindeer from Finland (Laaksonen et al., 2010, 2015). Pathological impacts caused by *R. andersoni* are not well characterized, but inflammatory changes within ruminal vessels have been reported (Kutz et al., 2018; Laaksonen et al., 2015). The arterial worm, *E. schneideri*, and the legworm, *Onchocerca* spp., can cause more apparent pathology in infected ungulate hosts, including moose (Benedict et al., 2023; Pledger, 1978; Verocai et al., 2012). The microfilariae of *E. schneideri* and *Onchocerca*, however, are found in the skin of their mammal hosts, instead of circulating in the blood (Anderson, 2000; Benedict et al., 2023; Pledger, 1978; Verocai et al., 2012). Therefore, definitive diagnosis may require either isolation of adult specimens at necropsy or skin biopsies for detection of microfilariae or subcutaneous adult nematodes.

The detection of filarioid nematodes of moose whose microfilariae are found circulating in blood, including *S. yehi* and *R. andersoni,* is most often achieved using classical, microscopy-based techniques, such the modified Knott's test on fresh whole blood (Grunenwald et al., 2016). Despite its relatively high specificity, the modified Knotts's test presents a limited sensitivity, and early-stage infections are rarely diagnosed. In addition, false negative results may be common due to the synchronous nature of microfilariae presence relating to circadian rhythms and seasonality (Grunenwald et al., 2016). Furthermore, modified Knott's test requires that whole blood samples be refrigerated and shipped in chilled boxed to laboratories for analysis. For wildlife captures in remote areas, this can be a challenge. Additionally, only using fresh whole blood precludes analyzing any archived whole blood samples that are frozen. Alternative diagnostic methods include the recovery of adult worms from the peritoneal cavity at post-mortem examination, followed by morphological and/or molecular examination. Molecular approaches have increasingly been utilized in studies to elucidate the biodiversity and epidemiology of parasitic nematodes infecting moose and sympatric ungulates (Kutz et al., 2007; Verocai et al., 2014a, 2014b, 2020). Among the advantages of such methods are their higher throughput and implementation in retrospective studies utilizing archival samples.

Infections by *S. yehi* in moose, similar to other wild cervids, can lead to peritonitis (Kutz et al., 2012; Laaksonen et al., 2009). In fact, bacterial peritonitis attributed to *S. yehi* was reported in a collection of nine moose calves from Alaska and was associated with high microfilaria counts and mortality (Kutz et al., 2012). Additionally, a related species, *Setaria tundra* (Issaitshikoff and Rajewskaya, 1928) was considered the cause of peritonitis outbreaks in young reindeer from Finland (Haider et al., 2018; Laaksonen et al., 2007, 2010; Nikander et al., 2007). The recent increasing concern of *Setaria* infection in northern ungulates has prompted the need to develop more sensitive and specific techniques to detect parasitic infection. Therefore, the aim of this study was to validate a qPCR method for detection of *S. yehi* DNA in blood samples of moose. Furthermore, we compared modified Knott's test results from fresh and frozen moose blood samples to quantify *S. yehi* and *R. andersoni* from archived frozen samples.

2. Materials and methods

2.1. Sample collection

All procedures for care, handling, and experimentation were approved by the Alaska Department of Fish and Game Division of Wildlife Conservation Animal Care and Use Committee (protocol 0046 and 0086).

During the winters of 2019–2022, we captured moose within Alaska Department of Fish and Game – Game Management Unit 15, on the western Kenai Peninsula, Alaska, USA. We caught adult cow moose in either November ($n = 46$) or March ($n = 54$), while we caught 10-month-old calves in March (males: $n = 32$; females: $n = 31$). Moose were chemically immobilized by aerial darting, using 1–3 cc powder dart filled with a combination of thiafentanil oxolate (0.03 mg • kg-1 estimated body mass; 10 mg • mL-1; ZooPharm LLC, Laramie, WY, USA) and xylazine HCl (0.20–0.26 mg • kg-1 estimated body mass; 100 mg • mL-1; Lloyd Laboratories, Shenandoah, IA, USA). The immobilization drugs were antagonized with naltrexone HCl (10 mg mg^{-1} thiafentanil; intramuscular; 50 mg mL^{-1}; ZooPharm) and atipamezole HCl (0.02–0.04 mg kg^{-1} estimated body mass; ¼ dose intravenous, ¾ intramuscular; 25 mg mL^{-1}; Zoetis, Parsippany, NJ, USA). Blood was collected from jugular venipuncture into 6 ml plastic trace element whole blood tubes (K_2 EDTA; BD Vacutainer® PN#368381; Becton, Dickinson, and Company). Prior to microscopical analysis, we stored whole blood samples at either 2 °C (March samples) or frozen at −20 °C (November samples).

In February 2020, during a necropsy of a moose calf at the Kenai Moose Research Center, adult filarioid nematodes were recovered from the peritoneal lining of the gastrointestinal tract and the pericardium and stored in 100% ethanol until morphological and molecular identification.

2.2. Modified Knott's test

Fresh blood was analyzed using a modified Knott's technique (Lucio-Forster and Lejeune, 2021), identifying the density of *S. yehi* and *R. andersoni* per mL of blood. The remaining fresh blood (~4 – 5 ml) was then frozen, thawed, and analyzed following the same procedure along with the frozen blood collected in November. A 0.1% methylene blue dye was added to the sediment, then the material transferred onto microscope slide, and microfilariae were counted and morphologically identified to genus or species-level. Microfilariae of *Setaria* are sheathed, have a round and blunt anterior extremity and a tapered posterior extremity. Specifically, *S. yehi* microfilariae are 285–315 μm long, and of 5–7.5 μm wide (Grunenwald et al., 2016). Microfilariae of *R. andersoni* are also sheathed but have a blunt anterior extremity and a rounded posterior extremity, measuring 140–169 μm long and 4–5 μm (Lankester and Snider, 1982). The number of *Setaria* and *Rumenfilaria* microfilariae per mL of blood (microfilaremia) was determined for each sample. The

remaining blood samples were frozen at −80 °C until molecular examination.

2.3. Adult nematode identification and sequencing

The filarioid nematodes collected from peritoneal cavity and pericardium of the moose calf were morphologically identified as adult female *Setaria yehi*. Specimens were sectioned, and fragments of the midbody were subjected to DNA extraction using the QIAGEN DNeasy® Blood & Tissue kit (QIAGEN®, Hilden, Germany) per manufacturer's instructions. The extracted DNA was kept frozen at −20 °C until further analysis was performed. As per Kulpa et al. (2021), previously published primers were used to amplify the cytochrome oxidase c subunit 1 (*cox1*) gene region of filarioid DNA using a conventional polymerase chain reaction (cPCR) approach (Table 1). PCR was performed in a 25 μL volume master mix consisting of 1x GoTaq® Green Master Mix (Promega Corporation, Madison, WI, USA), 0.625 μL of each primer at 0.25 μM concentration, and 1 μL of DNA template. Cycling conditions included an initial denaturation step at 95 °C for 2 min followed by 40 cycles of 95 °C for 45 s, 52 °C for 45 s, and 72 °C for 90 s, with a final extension step at 72 °C for 5 min. Amplification of *cox1* was confirmed via agarose gel electrophoresis of amplicons and purified with E.Z.N.A. Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA), following manufacturer's protocol. Sequencing of the purified products occurred in both directions using original PCR primers at Eurofins Genomics (Louisville, KY, USA).

2.4. Primers and probe for qPCR

The generated 649 bp sequence fragments of the *cox1* (GenBank Accession No PQ196581– 83) gene was utilized for designing of primers and probe. Partial *cox1* sequences of *S. yehi* were aligned with homologous sequences of spcies of *Setaria* available through GenBank and closely related genera of filarioid nematodes that may infect moose using MEGA X 10.1 (Kumar et al., 2020). Forward and reverse primers and probe were designed using PrimerQuest™ tool (Integrated DNA Technologies, IDT) to amplify a 121 bp region that allowed species-specificity and to prevent false positive (Table 1). Primers and probe were purchased from Thermo Fisher Scientific and stored at −20 °C after suspension in nuclease-free water.

2.5. TaqMan probe-based qPCR

Genomic DNA was extracted from 200 μL of blood using the QIAGEN Mini Blood Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions. Amplification of the *cox1* region of *S. yehi* was performed via qPCR. Reactions were carried out in a volume of 20 μL containing 0.5 μL of 20 μM probe, 0.5 μL of 50 μM forward and reverse primers, 10 μL of TaqMan Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts) and 5 μL of DNA template. Positive (DNA of *S. yehi* adult) and negative (nuclease-free water) controls were included in each assay. The reactions were performed in a MicroAmp™ Optical 96-Well Reaction Plate (Applied Biosystems™, Waltham, Massachusetts) and cycling was performed in the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts). Cycling conditions included an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. To determine the primer efficiency and detection threshold, serial 10-fold dilutions of positive control DNA were performed in triplicate and threshold cycle (Ct) values were recorded (Fig. 1). Data of amplification, cycle threshold (Ct) values, and mean Ct were recorded for each run. Ct values of serial dilutions of positive control were plotted against the log of the sample quantity to create a standard curve. The slope of the regression between the log values and average Ct value collected from qPCR results was calculated using the following equation: $Efficiency\,(\%) = \left(10^{\frac{-1}{slope}} - 1\right) x\,100$.

Table 1
Primers and probe used for identification of adult *Setaria yehi* (conventional PCR) and microfilariae (qPCR).

	Primer name	Sequence
Setaria yehi adult	COINT-F	TGATTGGTGGTTTTGGTAA
	COINT-R	ATAAGTACGAGTATCAATATC
Microfilariae	Syehi-F	TGGTGGACCTGGTAGAAGT
	Syehi-R	CCCTAACAAAGAACCAATACCAAC
	Probe	6FAM-AAGGGTTGAAGGTCAGCCTGAGTT-MGB

2.6. Validation of qPCR

To validate the qPCR for detection of *S. yehi*, we assessed the primer efficiency, dynamic range, and specificity of the reaction. The dynamic range and primer efficiency was determined using a 10-fold dilution series of genomics DNA of adult *S. yehi*. The qPCR assay exhibited excellent linearity between the log of DNA concentration and Ct values over 4 orders of magnitude (Fig. 1). Detection threshold was determined to be 0.157 pg/μL with Ct value of 36. Using the slope of the standard curve, the primer efficiency was calculated to be 93.63%. The qPCR assay did not detect *R. andersoni* DNA in control blood samples.

2.7. Data analysis

We used simple linear regression to compare microfilaria densities between a subset of fresh and frozen blood samples ($n = 112$; STATA version 15.0; StataCorp LP, College Station, Texas, USA). Kappa value was used to estimate the agreement between modified Knott's test and qPCR (McHugh, 2012).

3. Results

3.1. Modified Knott's results

The results of modified Knott's test and qPCR are shown in Table 2.

3.2. Comparison of microfilaremia counts using fresh and frozen blood

Out of the 112 fresh blood samples, 75 samples contained densities of *S. yehi* (range: 2–14,490 *S. yehi*/mL blood), while 27 samples contained densities of *R. andersoni* (range: 2–5071 *R. andersoni*/mL blood).

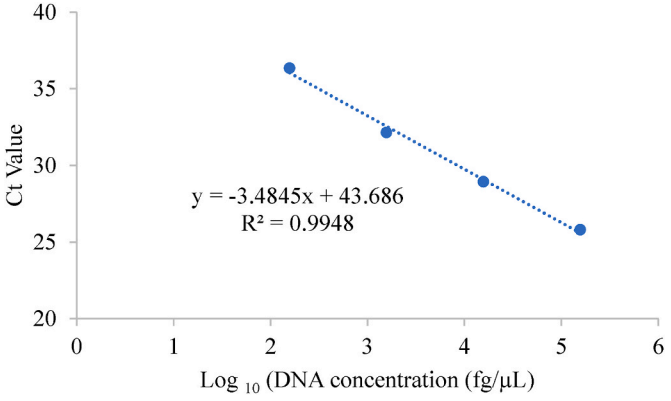

Fig. 1. Standard curve of qPCR for detection of *S. yehi*. This curve was created by plotting average Ct values across three replicates using 10-fold serial dilutions of parasite genomic DNA.

Table 2
Summary results of moose blood samples screened for *Setaria yehi* using modified Knott's test and qPCR.

	Total number of samples	MK results		qPCR result	
		MK Positive	MK negative	qPCR positive	qPCR negative
2019	17	6	11	4	13
2020	88	41	47	43	45
2021	27	0	27	0	27
2022	34	7	27	7	27
Total	166	54	112	54	112

Excluding fresh blood samples that contained no filarioid nematode microfilariae and outliers outside of the 95th percentile of the data (*S yehi* $n = 4$; *R. andersoni;* $n = 2$), we then regressed the fresh blood sample density of each filarioid nematode against the frozen blood sample density. *Setaria yehi* density in fresh blood was highly corelated to frozen blood ($n = 71$; $F_{1,69} = 315.32$, $P < 0.001$, $R^2 = 0.82$; Fig. 2A). *Rumenfilaria andersoni* densities were correlated considerably less than *S. yehi* ($n = 41$; $F_{1,39} = 32.37$, $P < 0.001$, $R^2 = 0.45$; Fig. 2B).

3.3. Comparison of Modified Knott's and probe-based qPCR for detection *S. yehi*

The results of modified Knott's test and qPCR is shown in Table 2. Overall, out of 166 fresh whole blood samples, 54 tested positive for *S. yehi* using modified Knott's test, with an average count of 472.17 microfilaria/mL (0–14,490 mf/mL). Additionally, 54 samples tested positive via qPCR. When both tests were combined, a total of 62 individual samples (37.4%) tested positive for *S. yehi* (Table 2). When modified Knott's and qPCR results were compared for each sample, 46 of 62 samples had concordant positive results (Table 2). A total of 16 samples had discordant Knott's and qPCR results, with 50% testing Knott's positive and qPCR negative and the remaining 50% testing Knotts negative and qPCR positive. The agreement between Knott's test and qPCR results was estimated by kappa value. Overall, the observed agreement was 150 samples out of 166 total samples, making the kappa = 0.9 (Table 3).

4. Discussion

Filarioid nematodes are common and often pathogenic parasites of wild North American cervids, including moose (Benedict et al., 2023; Grunenwald et al., 2016; Kutz et al., 2012; Verocai et al., 2012). The unprecedented pace of climate change is drastically affecting the ecology and health of wild ungulates, in particular those distributed at higher latitudes (Kutz et al., 2014).

Table 3
Summary results of modified Knott's (MK) test and qPCR for *Setaria yehi*. Kappa value (0.9) of modified Knott's test and qPCR results showed very good agreement between two tests.

	MK Positive	MK Negative	Total
qPCR Positive	46	8	54
qPCR Negative	8	104	112
Total	54	112	166

The biology of arthropod vectors is intimately influenced by climatic factors. Direct and indirect impacts of insect harassment may also be exacerbated, further impacting health and wellbeing of ungulate hosts (Benedict and Barboza, 2022; Benedict et al., 2024). Moose are especially affected by dipteran insect harassment, including mosquitoes, black flies (Simuliidae), horse and deer flies (Tabanidae), and the moose fly, *H. alcis* (Muscidae) (Lankester and Sein, 1986; Benedict and Barboza, 2022). Among the indirect impacts of blood-feeding insects on ungulate hosts is the transmission of filarioid nematodes, which may, to some extent, increase in incidence due to ungulate geographic range expansion, including the white-tailed deer, in the following decades (Tape et al., 2016; Dawe and Boutin 2016), which is another susceptible host for for *S. yehi* and *R. andersoni*.

Infections by *S. yehi* and *R. andersoni*, including co-infections, are relatively common in moose from Alaska, and other regions in North America (Dullen and Beckmen, 2006; Grunenwald et al., 2016; Kutz et al., 2012). Infections by *S. yehi* are of special concern due to its more pronounced pathogenicity and reports of disease outbreaks associated with the infection by the congeneric *S. tundra* in reindeer from Finland (Laaksonen et al., 2007; Laaksonen et al., 2017; Laaksonen et al., 2007; Paulsen et al., 2017). While the impact of *S. yehi* on an individual cervid host may range from subclinical to fatal cases of peritonitis (Kutz et al., 2012), its impact on herd health and host population dynamics remains unclear.

It is noteworthy that many historical reports have identified the abdominal nematodes of wild cervids to genus-level or species identification was presumed based on the prevailing knowledge at the time (Cowan, 1946; Erickson and Highby, 1942; Hoeve et al., 1988; Wünschmann et al., 2015). In moose, *S. yehi* has been reported across its range, but it also reported in areas of North America where moose is absent, and *Odocoileus* spp. are present (Anderson, 1962; Becklund and Walker, 1969; Grunenwald et al., 2016; Hoeve et al., 1988; Kutz et al., 2012; Prestwood and Pursglove, 1977; Samuel, 1967; Tran et al., 2022; Walker and Becklund, 1970; Wünschmann et al., 2015).

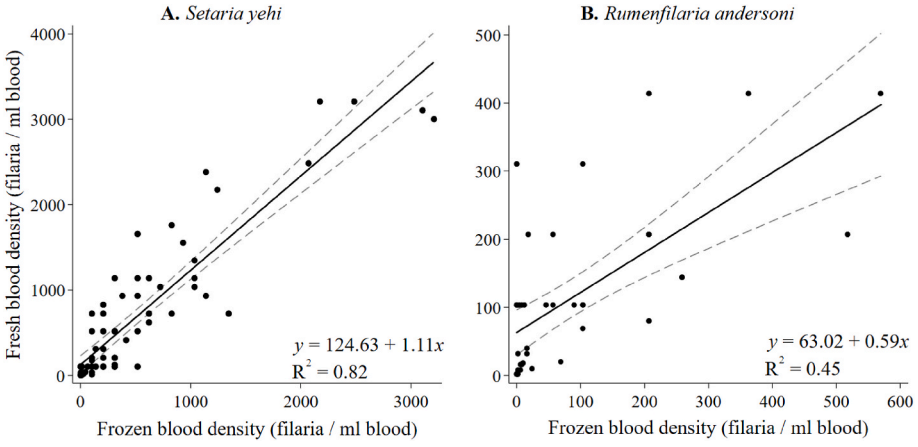

Fig. 2. Modified Knott's technique results comparing A. *Setaria yehi* and B. *Rumenfilaria andersoni* microfilariae counted from the same sample of fresh and frozen moose blood collected from wild moose on the Kenai Peninsula, Alaska, USA.

4.1. qPCR is a valid method for detecting S. yehi

The results of the kappa statistic indicate almost perfect agreement, according to Everitt (1989). This means the that our novel probe-based qPCR test, as compared to the previously validated modified Knott's test, showed very similar results for each sample. Thus, we can confidently say that our qPCR method is accurately identifying *S. yehi* in blood samples. Moreover, the qPCR assay did not amplify *R. andersoni*, another common filarioid nematode found in moose blood, confirming that the assay is specific for *S. yehi*.

While the agreement between the two tests is not perfect, one could choose to implement these in combination for increased diagnostic sensitivity, accounting for samples with discrepant, false-negative results. Among reasons for potential false-negative qPCR results are erroneous homogenization of thawed blood samples, true absence of microfilariae in the blood volume used for DNA extraction, and other technical errors. Another plausible reason for *Setaria*-positive blood samples via modified Knott's and *S. yehi*-negative qPCR, would be the presence of *S. yehi* haplotypes that may not be detectable with the designed primers and probe or the presence another *Setaria* species circulating in moose in Alaska. The qPCR assay was designed to be specific to *S. yehi*, and not detect other species, especially *S. tundra*. Although *S. tundra* has never been unequivocally reported in North America, the introduction of semi-domesticated reindeer from Eurasia into Alaska over a century ago (Finstad et al., 2006; Verocai et al., 2013), could have allowed for *S. tundra* introduction and subsequent establishment in North American *R. tarandus*, and colonization of other sympatric, susceptible ungulate hosts. To date, the only records of *Setaria* in domestic reindeer from Alaska, and North America, was attributed to *S. yehi* (Dieterich and Luick, 1971). Screening reindeer, caribou, and moose from Alaska is warranted to investigate the potential presence of *S. tundra* in sympatry with *S. yehi*, and the possibility of co-infections.

4.2. Benefits to qPCR over Modified Knott's test

Our novel qPCR design has several advantages over modified Knott's test, such as species-specific identification, faster diagnosis, and increased testing capability. Morphologic identification of adult or larval *Setaria* requires a significant time investment in training and sample examination. Very few diagnostic laboratories have the capability of performing Knott's test on an entire herd of animals due to time restraint and knowledge of morphological identification of microfilaria, but many laboratories have access to qPCR and DNA extraction kits. Therefore, the development of this *Setaria* specific qPCR will allow for increased capability of testing.

4.3. Future explorations

In addition to rapidly identifying *Setaria*, one could also apply this technology to diagnosing other pathogens of interest. In an unpublished study, it was found that moose infected with *S. yehi* are commonly coinfected with other filarial species such as *R. andersoni*, whose *in vivo* diagnosis seem even more challenging than that of *S. yehi* (Grunenwald et al., 2016). The promising results of this study indicate that the qPCR can be adapted to detect other pathogens in a separate simplex or multiplex qPCR for the detection of co-infections.

As *S. yehi* is a multi-host filarioid nematode, reported from various wild cervid species distributed across North America (Becklund and Walker, 1969; Kutz et al., 2012; Prestwood and Pursglove, 1977; Weinmann et al., 1973), our probe-based qPCR assay is directly applicable for diagnostics and epidemiological surveillance across their range. Establishing baseline *S. yehi* prevalence across different cervid hosts and geographic areas is relevant for monitoring the impact of climate change on transmission dynamics (Kutz et al., 2014; Laaksonen et al., 2010).

In addition, this qPCR protocol would allow more efficient screening of mosquito vectors for *S. yehi* in comparison to conventional PCR approaches (Tran et al., 2022). Nevertheless, additional optimization steps in sample preparation may be necessary to mitigate PCR inhibitors known to be present in arthropod vectors (Gopal et al., 2012; Murray et al., 2015; Schrader et al., 2012). Increased screening of potential vectors across the range of distribution of *S. yehi* may reveal novel vector-associations, which are directly informative for understanding transmission ecology.

4.4. A tool for assessing life-cycle parameters and seasonality in definitive hosts

The application of this qPCR assay on serial sample collections of a group of animals across time could provide data for elucidating life cycle parameters of *S. yehi* that have not been well-established. This includes the pre-patent period and potential differences among cervid host species, trends associated with host age and seasonality, and different modes of infection (Alhassan et al., 2015; Evans et al., 2022; Kronefeld et al., 2014; Sobotyk et al., 2022). While nearly all *Setaria* infections of a definitive host will follow the transfer of L3 via mosquito bite, and the individual host that was blood fed on will develop the infection, L3 may migrate to the fetus of a pregnant female susceptible host. Congenital or prenatal infection has been demonstrated in *S. yehi* in Columbia black-tailed deer (*O. h. columbianus*) (Weinmann et al., 1973) and other *Setaria* species, including *Setaria marshalli* and *Setaria digitata* of cattle from Japan and South Korea, East Asia, (Fujii et al., 1995; Kim et al., 2010; Shoho, 1965; Wee et al., 1996) and *Setaria bidentata* in red brocket deer (*Mazama americana*) from Peru, South America (Gomez-Puerta and Mayor, 2017). Nevertheless, while proven, the epidemiological relevance of prenatal transmission of *S. yehi* has been questioned (Weinmann and Shoho, 1975). As a multi-host parasite, there is a need for elucidating this mechanism further, including potential differences among host-species.

5. Conclusions

Based on the high primer efficiency, low detection threshold for DNA on qPCR, and high kappa statistic when comparing the results from modified Knott's test and qPCR test, we can conclude that this novel qPCR is an accurate and effective method for molecularly identifying circulating microfilariae in moose blood samples. With this faster and less labor-intensive test, we aim to increase rate of testing to further understand trends in parasite movement and prevalence. By further understanding these factors, more information can be gathered on the pathology of *S. yehi* infections and insights into methods for preventing further moose population decline. Additionally, both fresh and frozen blood samples can be analyzed quickly using the qPCR methods, and those samples that test positive then can be analyzed via the modified Knott's test for quantitative analysis.

CRediT authorship contribution statement

Guilherme G. Verocai: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jordan L. Gomez:** Writing – original draft, Formal analysis. **Hassan Hakimi:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Matthew R. Kulpa:** Writing – review & editing, Methodology, Formal analysis. **Joe L. Luksovsky:** Writing – review & editing, Methodology. **Daniel P. Thompson:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **John A. Crouse:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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