Spring 5-9-2020

An Investigation of the Practical Challenges to Using Molecular Genetic Techniques to Identify the Parasite Burdens of Vertebrate Animals from Non-Invasive Sampling

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An Investigation of the Practical Challenges to Using Molecular Genetic Techniques to Identify the Parasite Burdens of Vertebrate Animals from Non-invasive Sampling

By

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A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford

2020

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ACKNOWLEDGEMENTS

First, I would like to acknowledge my advisor, Dr. Richard Buchholz for advising me throughout this research process and providing me with the materials and the guidance needed to create a successful thesis project. I would also like to thank Dr. Rebecca Symula and Dr. Glenn Parsons for their time and consideration they put forth as my second and third readers, and Dr. Susan Balenger for helpful comments on my thesis. I would also like to thank Victoria Monette and Alaina Skidmore for assisting me in the lab and being constant encouragement and supporters throughout the entire process. I am very grateful to my parents for constantly pushing me to work my hardest and try my best in my lab work. Lastly, I would like to acknowledge the Sally McDonnell Barksdale Honors College for giving me the opportunity to participate in the thesis project.

ABSTRACT

KEELY COX: An Investigation of the Practical Challenges to Using Molecular Genetic Techniques to Identify the Parasite Burdens of Vertebrate Animals from Non-invasive Sampling

(Under the direction of Dr. Richard Buchholz)

Molecular genetic techniques have become popular methods in ecology and wildlife conservation research. Advances in molecular genetic methods, particularly PCR (polymerase chain reaction), make it possible to amplify the numbers of specific DNA sequences from a sample with only a few original copies. Theoretically, the specificity of this approach should make it possible for wildlife biologists to identify and quantify the parasite and disease burden of endangered animals without being limited by the rarity of collaborators with expertise in the taxonomy of obscure parasite taxa. Because PCR requires just a small amount of DNA, the added benefit of a molecular genetic approach is that non-invasive sampling methods can be used that do not harm the endangered animals. My research involved two related projects. First I attempted to separate, morphologically identify, sequence, and isolate DNA from nematodes present in fecal samples collected non-invasively from the Baird's tapir in Belize. This attempt was not successful but I did discover several practical obstacles to this type of work that made me wonder about the success rate of molecular parasitology in other studies of wildlife species. The second project was a systematic review of the literature of the practical challenges associated with using molecular genetic methods to identify parasitic nematodes in fecal samples of domestic and wild animals, and humans. This literature review led me to the conclusion that non-invasive sampling methods are only beneficial

when the sequence of the studied nematode has already been named through prior research.

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CHAPTER ONE

An Attempt to Identify Nematodes in the Feces of Baird's Tapir Using A Standard Protocol for Lysis, Direct PCR, and Sequencing

Introduction

The Earth's natural habitats and the wildlife in them are in decline, leading to an extinction crisis (Pellens, 2018). As the human population continues to grow exponentially (Hubbert, 1996) our unsustainable use of resources poses various threats to other species. Some of these threats are obvious, such as direct harvest and persecution of species for food, recreation and trade (Pellens, 2018). Other threats are indirect or insidious in that they act as stressors that subtly reduce lifetime fitness of individual animals by impairing reproduction and/or reducing survival (Balestri, 2014). These stressors can include anthropogenic contaminants (Frena, 2016), reduction of food availability due to habitat degradation by humans (Tylianakis, 2007), and frequent non-consumptive disturbance by humans (Burgin, 2015) such as from ecotourists, hikers and off-road cyclists.

Anthropogenic stressors may have cumulative effects that occur on top of naturally occurring stressors such as intraspecific competition for food and mates (Munns, 2006), dominance interactions (Sheldon, 1996), pursuit by predators (Munns, 2006) and infection by parasites (Sheldon, 1996). The negative health effects of infections by parasites and other disease organisms on threatened and endangered species are steadily becoming a focus of attention for conservation biologists (Gomez, 2013).

When species decline to low population size in a reduced or scattered geographic range, the effects of parasites, when combined with the effects of other stressors, can lead to the reduction in resistance or tolerance to certain infections, possibly contributing to the risk of extinction. In certain areas of the Mojave desert, upper respiratory tract disease incurred by the bacteria *Mycoplasma agassizii* in the desert tortoise (*Gopherus agassizii)* has contributed to significant population decline in the tortoises (Hunter et al., 2008). Another example of decline in wildlife populations due to parasites or disease is seen in the California sea otter (*Enhydra lutris nereis)*. *Toxoplasma gondii*-associated meningoencephalitis is a disease that is responsible for 72% of otter infections and 16% of mortality in beachcast sea otter carcasses, and is the main cause of the California sea otter's population decline; making them a threatened species (Miller et al., 2004).

Parasites use host resources to thrive and reproduce; lowering host fitness to varying degrees (Sheldon 1996). Parasitic worm-like species in the phylum Nematoda are well known for their harmful effects on domestic animals, such as heartworms and lungworms in cats and dogs (Traversa, 2010) and habronemosis in horses (Saeed, 2019), for example. Less commonly known is that parasitic nematodes can also harm humans and wildlife species. Hudson (1986) found that the nematode *Trichostrongylus tenuis* was associated with poor breeding success in the red grouse (*Lagopus lagopus scoticus)*, causing a decline in this bird species' population. Another example is the human parasitic threadworm, *Strongyloides stercoralis,* that infects around 100 million people worldwide; causing gastrointestinal distress in healthy individuals, but can be fatal in those who are immunocompromised (Becker, 2015).

Because wild species are free-living, the effects of nematode infection on their health is difficult to study and little is known on this subject, compared to veterinary understanding of parasitic impact on domesticated species. Nevertheless there is limited evidence that stressors can affect the intensity of parasitic infection. In the red grouse, low food availability was a stressor that caused a higher infection rate by *Trichostrongylus tenuis* (Hudson, 1986). However, such stress effects are not always straightforward. For example, chronic stress in the capybara (*Hydrochoerus hydrochaeris)*, a South American rodent, causes a higher infection by some specific helminths and protozoans like *Strongyloides chapini* and *Eimeira hydrochoerid*, but not other parasite species (Eberhardt, 2013). This study suggests a complex relationship between stress, the physiological cost of immunity, and the damage of different types of parasitic infections.

A high threat of parasitic disease (Cable, 2017), and the greatest loss of biodiversity (Ahumada, 2011) is occuring in tropical forests. Species richness of nematodes in tropical rainforest is 300% more than that in the temperate rainforest areas (Porazinska, 2010). Deforestation in the tropics increased 53% between 2001 and 2012 from an average of 6,000 kilo hectares during the first half of their research period to 9,200 kilo hectares during the latter half (Austin et al., 2017). Due to this deforestation, anthropogenic stressors are driving species loss at a rate 100 times faster than previous base levels (Austin et al., 2017). These findings further support that we may be entering a sixth era of mass species loss on a global scale (Ceballos et al., 2015).

In order to investigate how anthropogenic disturbance in the tropics may be contributing to the loss of biodiversity, I attempted to identify which parasitic nematode

species are found in a population of the endangered Neotropical mammal, Baird's tapir (*Tapirus bairdii)*, by isolating DNA from fecal nematodes and amplifying and sequencing their DNA. Monette (2019) used molecular methods to identify 4 individual nematodes from one of the 6 morphotypes that she recovered from field-collected fecal samples. This first chapter of my thesis provides an overview of my unsuccessful attempt to identify additional nematodes morphotypes from these same fecal samples.

Materials and Methods

A. Study Species and Study Area

Tapirs are one of the last members of the Neotropical megafauna. They are shy herbivores living in habitats that vary from deciduous forests to tropical rainforests in Central and South America as well as in Southeast Asia. Tapirs can be found anywhere from sea level to heights of at least 3,350 meters. The four species of New World tapir are as follows: the Baird's tapir (*Tapirus bairdii),* the mountain tapir *(Tapirus pinchaque),* the lowland tapir *(Tapirus terrestris),* and the Malayan tapir *(Tapirus indicus)* (The New Encyclopedia Britannica, 2010).

Tapirs are of conservation concern because they require large, inter-connected forested areas with protection from over-hunting to persist (Naranjo 2018). The endangered Baird's tapir used to occur from Mexico to northern South America (Garcìa-Marmolejo et al., 2015) but now that range has been reduced by 50% in just three decades (Schank et al., 2015). These tapirs have critical ecological roles that help shape the Neotropical forest community (Jorge et al., 2013). They disperse the seeds of rainforest plants, support dung beetle diversity, and create depressions in the soil for wallowing, thereby creating pools that can be used by other animals (Garcìa et al., 2012, Garcìa-Marmolejo et al., 2015, O'Farrill et al., 2013).

The tapir is an ecosystem engineer that serves as a great source of seed dispersal of trees whose fruits are in their diet. Because of this behavior, tapirs provide an ongoing food and shelter supply for other animals in their region (O'Farrill 2011). The populations of Baird's tapir (*Tapirus bairdii)* have been reduced drastically because of

habitat fragmentation caused by deforestation, constraining the population of this species to protected areas (Carillo 2019).

B. Sample Collection Methods

Baird's tapir fecal samples were collected in North Western Belize by Monette (2019), who provides a detailed description of the study site and sampling methodology. I was not involved in the field collections, but provide a brief summary below, before describing how I assisted in sorting and processing nematodes collected from the preserved feces.

The samples were found on trails that were covered by a camera survey grid that was used as a transect. For reach site where tapir fecal samples were found, GPS coordinates were recorded. Each transect was hiked a second time to collect any new feces that had been dropped. After collection of the fecal samples, they were homogenized, strained using purified water, and allowed to settle for three hours. The supernatant was poured out and the remaining sediment was stored in three different ways. Monette (2019) immediately isolated fecal DNA from each sample using a fecal DNA extraction kit. For my lab research, this DNA served as the template DNA for my standard positives for each PCR that was run on the nematode DNA. The rest of the feces from each field collection were divided between storage tubes for preservation in 95% ethanol and 10% formalin.

Each of the forty-four samples preserved in the formalin and ethanol were centrifuged in an IEC HN-SII Centrifuge at 2000 rpms for ten minutes in order to concentrate the sediments at the bottom of the tube. After centrifugation, the sediment of

the sample was poured into a petri dish to be examined under a Labomed Luxeo 4Z Stereozoom dissecting microscope (10x magnification). All of the nematodes found in the dish were pipetted into a vial and marked with the number of nematodes found.

C. Sorting nematodes by morphology

I poured the vials containing the nematodes into a petri dish along with water purified by reverse osmosis (RO). The dish with the RO water and ethanol was set aside for about 15 minutes to allow the nematodes to settle to the bottom of the petri dish. Once settled, the nematodes that were in the vial had moved to the bottom of the petri dish. I examined the dish for nematodes at 10x magnification under a LaboMed Luxeo 4Z Stereozoom dissecting microscope.

Monette (2019) identified six main types of nematode morphology present in the ethanol samples. Morphology A, B, and C were crimson colored nematodes. Morphology A contained a mouth protrusion, morphology B had no mouth protrusion, and morphology C had a thicker body type. Morphology D, E, and F were clear and larger nematodes. Morphology D did not have a whip-like tail, morphology E had a whip-like tail and a smooth body type, and morphology F had a whip-like tail with ridges on the outside of the body. These different morphology types are represented in **Table 1**. Using a pipet, each nematode was extracted from the dish and placed in its own vial with ethanol. Each vial was labelled with morphological type. After separating every nematode, the case with the vials was properly labelled and placed into a freezer.

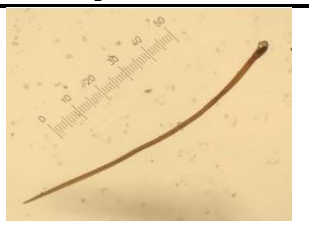
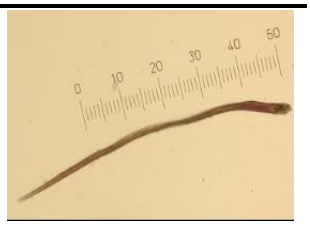
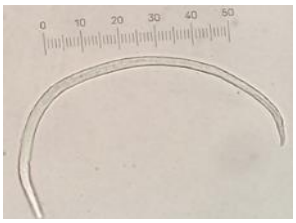
Morphology Type	Description	100x magnified photo
A	Long and slender, amber-colored body with a clear mouth protrusion; smooth body	
B	Long and slender, amber-colored body with no mouth protrusion; smooth body	
C	Tear shaped, amber-colored body with mouth parts at the wider end of the body; body cavity near the mouth; smooth body	
D	Clear body with a short tail, smooth body	
E	Clear body with a long tail, smooth body	
F	Clear body with long tail; ridges on side of the body	

Table 1. A description of the six different nematode morphotypes that were present in formalin and ethanol preserved fecal samples from the Baird's tapir. The scale lines in each photo represent 10 μm. (Used with permission from Monette (2019)).

D. Parasite DNA Analysis

Lysis:

For lysis, a few individuals from each of the A-C and F morphologies were selected to be lysed. A nematode was mouth- pipetted out of the vial and placed into a strip tube and properly labeled. Because nematodes can be lost during the transfer process, each tube was inspected microscopically to confirm the presence of a nematode. The tubes were left open, or heated in an Eppendorf AG Mastercycler gradient thermal cycler (No. 533113946) in order to evaporate any leftover ethanol that was dispensed into the strip tubes because ethanol will inhibit cell lysis. Following the methods of Chalasani (2016), a lysis master mix was made containing 0.3 microliters of Viagen proteinase K (Cat# 102-T) and 19.7 microliters of Viagen DirectPCR (mouse tail) per worm. In some lysis trials, more Proteinase K was used than recommended by Chalasani (2016), but the total amount of lysis solution used remained at 20 microliters per worm. Twenty microliters of lysis master mix was added to each tube of the PCR strip tube containing a nematode, incubated at 55 degrees Celsius for 16 hours, heated at 85 degrees Celsius for one hour, followed by refrigeration at around 4 degrees Celsius.

PCR:

A 23 µL PCR master mix solution for each nematode sample was prepared with the following components: 12.5 microliters of Promega GoTaq green master mix, 2x, 9.5 microliters of ddH_2O, 0.5 microliters of forward primer (18S), and 0.5 microliters of backwards primer (18s). The primers target the 18S rRNA region of the nematodes and the sequences are as follows: 5’ GGCGATCAGATA-CCGCCCTAGTT 3’ (18S 965

Forward) and 5’ TACAAAGGG-CAGGGACGTAAT 3’ (18S 1573R) (Powers, 2009). Two microliters of the lysed nematode solution (containing the DNA template) were added to each tube of PCR master mix. For the positive and negative PCR controls, two microliters of a positive control DNA sample (fecal sample) was added to the PCR master mix solution and two microliters of ddH_2O was added to PCR master mix for the negative control. Brief centrifugation of the PCR 0.2 milliliter strip tubes was completed before placing them in the thermal cycler.

During the PCR process in the thermal cycler, the three main stages that occurred were denaturing, annealing, and extending. Denaturing involved the heating of the double-stranded template of DNA at 95 degrees Celsius for 4 minutes in order to separate the strand into single strands. The temperature lowered to 55 degrees Celsius for 30 seconds to allow the specific primer to attach to the template DNA in the annealing process. Finally, the temperature was raised to 72 degrees Celsius for 6 minutes and the new strand of DNA was formed by the Taq polymerase enzyme. These three main stages of PCR were repeated forty times, which doubled the number of DNA copies every time the stages were repeated.

Electrophoresis:

Agarose gel electrophoresis was used to check the quantity and size of the fragments of DNA. To make an agarose gel for electrophoresis, 1.5 grams of agarose powder was added to a glass jar. Next, 100 milliliters of the electrophoresis gel buffer was added to the container. This mixture was heated in one-minute intervals until it approached boiling. After heating the mixture, two microliters of ethidium bromide were added and swirled in the jar to mix. This mixture was poured into the electrophoresis gel

tray (with plastic combs) and allowed to set for 15 minutes. After the gel set, the plastic combs were removed from the gel to reveal the wells in the gel. The gel and tray were placed in the electrophoresis machine and the buffer solution was poured into the machine until the gel was completely covered. Five microliters of FlashGel™ DNA Marker 100-4000 base pair ladder was added to the first well of each row. To inspect the quantity and quality of DNA produced by nematode lysis, a 7.5 µL total volume of 2.5 microliters of FlashGel™ loading dye (5x concentration) with 5 µL of the lysed nematode solution were mixed together by pipetting onto a Parafilm laboratory film strip. This mixture was then pipetted into the well in the agarose gel. The gel was electrophoresed for 30 minutes at 110 volts. The gel was then photographed under UV transillumination on an AlphaImager HP. The printed photographs of the gel did not result in clear bands, so the DNA isolation process was attempted again using more than one nematode per vial in the lysing stage. This process was repeated with two worms, three worms, and two halved worms in each vial. The reagents were adjusted according to the amount of worms placed in the vials; following the procedure from Chalasani (2016).

Results

No successful extraction of DNA from any of the studied worm morphotypes was evident even though varying the number of individual nematodes, halving them, and adding more proteinase K was performed (**Table 2)**. After the lysis step, it was very evident that the nematodes were still intact, but they were moved onto the PCR stage. During the first few months of my lab research (March 2019-September 2019), the positive controls amplified in each electrophoresis gel that was run, matching the expected base pair length of 646 bps, so it was evident that PCR failure did not occur (**Fig. 1**). In the latter months of my lab research (September 2019-December 2019), the positive control bands and a strong ladder were not visible on the gel photographs, from which I conclude that the electrophoresis gel was run for too long and the DNA fragment ran off the gel, or that PCR failed **(Fig. 2)**.

Nematode Morphological Type (after Monette 2019)	# of Nematodes (per individual PCR striptube)	**Protease Volume** (µL)	Outcome of PCR Product
A	1 worm	0.3 Prot. K per worm	Unknown
A	1 halved worm	0.3 Prot. K per worm	No + control band
A	2 worms	0.3 Prot. K per worm	Unknown
A	2 halved worms	0.34 Prot. K per worm	No + control band
A	3 worms	0.34 Prot. K per worm	Unknown
B	1 worm	0.3 Prot. K per worm	Unknown
B	2 worms	0.3 Prot. K per worm	Unknown
B	2 halved worms	0.34 Prot. K per worm	No + control band
C	1 worm	0.3 Prot. K per worm	Unknown
C	2 halved worms	0.3 Prot. K per worm	No + control band
F	1 worm	0.3 Prot. K per worm	Unknown
F	2 worms	0.34 Prot. K per worm	Unknown

Table 2. A summary of the DNA extraction methods, lysis reagents, nematode morphological types, number of nematodes used, and the outcome of each attempt to amplify DNA.

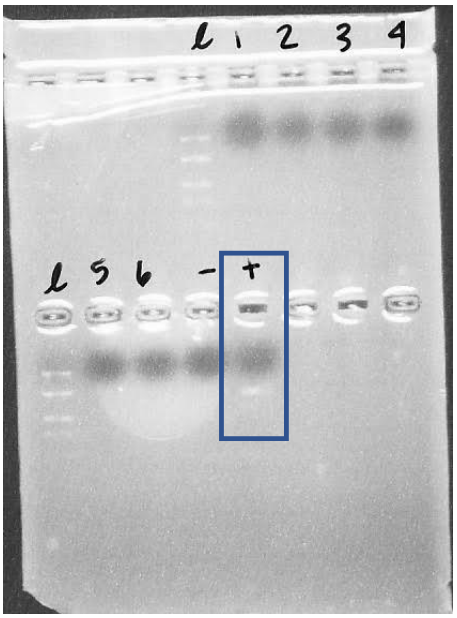


Fig. 1 A scanned photo of an electrophoresis gel image with a faint band on the positive control.

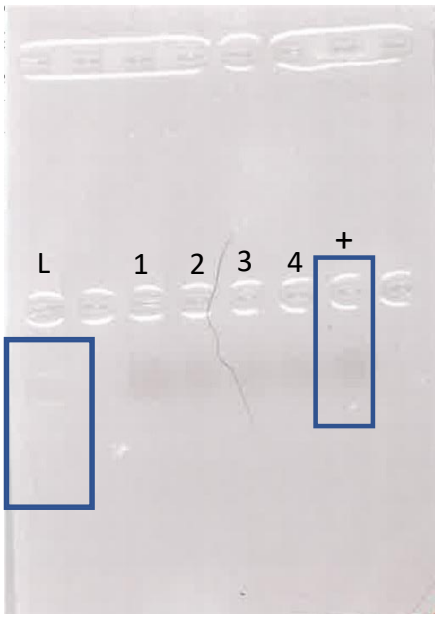


Fig. 2 A scanned photo of an electrophoresis gel image with a faint ladder, but no positive control band.

Discussion

The original goal of this study was to successfully isolate DNA from intestinal nematodes present in tapir fecal samples, and have them sequenced through Sanger sequencing. If enough DNA was successfully isolated and replicated, the DNA sequence of the nematode could have been found, and the nematodes present in the fecal samples would have been identified; however, this proved to be unsuccessful.

Throughout the process of attempting to replicate nematode DNA and receive distinct bands on an electrophoresis gel, I was faced with many challenges in acquiring the data that was needed to move on to the DNA sequencing step. Troubleshooting began at a pretty early stage in this research process. In regards to lysing the nematodes, prior research on the nematodes present in the Baird's tapir fecal samples was not available for reference when deciding which DNA extraction method to use. Although lysis is a common form of DNA extraction, the cell is not subjected to mechanical breakdown by, for example, centrifugation or grinding with glass beads. If the nematodes present in this study had hard coverings, the lysis reagents could have a tough time with penetrating through the worm and extracting the DNA (Reigstad, 2011). In the study completed by Monette (2019), she was only able to successfully lyse, run PCR, and electrophorese the non-parasitic nematodes of morphology D. She used the methods provided by Chalasani (2016) for lysing, which suggests that these methods may not be sufficient enough to penetrate the outer coat of the other nematode morphologies present in this study. If this research was to be repeated on morphologies A-C and F, it is suggested to perform some sort of mechanical disruption of the specimen (i.e. bead beating) prior to introducing a chemical/enzyme like proteinase K (Elkins, 2013).

In the beginning of my lab research, it was evident by the photographs of the electrophoresis gels completed in my study that there were no failures in PCR reagents and the thermal cycler operation because a positive control band was evident in each gel that was run. Some reasons behind the lack of clear bands in these gels could have been related to the time in which the gels were run in the electrophoresis machine. If the gels were run for too long, the bands of DNA could have run off the bottom of the gel. Towards the end of my lab research, the photographs of the gels showed that there was some sort of PCR failure because bands for the positive controls were no longer evident. This shift in seeing positive control bands to no longer seeing these bands could possibly mean I encountered malfunctions in lab equipment or spoiled PCR and lysis reagents.

In selecting the primers used for this research project, the same primers used in the research done by Monette (2019) were also used in replicating the DNA of the nematodes present in this study's fecal samples. A primer that targets the 18S rRNA region of nematodes was used because of the large number of 18S sequences that are available on GenBank, the presence of a 18S- based phylogenetic tree, and this gene's nature in ensuring a complete phylogenetic coverage of the phylum Nematoda (Powers, 2009). The same vial of reverse and forward PCR primers were used for the entire duration of this research project, so the lack of DNA amplification could have been caused by old primers. To troubleshoot this problem, it is suggested that fresh primer aliquots should be reconstituted or new primers should be obtained. Insufficient quantity of the PCR primer could have also led to poor replication/ amplification. In the gels that showed a positive control band was amplified, the absence of bands from the nematode

samples might be the result of insufficient template DNA for PCR to create a visible band.

The common problems associated with the use of the polymerase chain reaction to replicate DNA are mainly associated with reaction conditions, sequence accuracy, and amplification yield. The lack of amplification of DNA could possibly be caused by poor DNA integrity during DNA isolation, insufficient quantity of DNA, and complex targets (e.g. secondary structures). To troubleshoot these problems, DNA could be stored in a TE buffer to avoid degradation by nucleases, DNA polymerases with high sensitivity could be chosen for amplification, and the denaturation time and temperature could be increased to separate the double stranded DNA templates (Eggert, 2006).

The repetitive and meticulous troubleshooting of this lab research makes me question if it is feasible to expect widespread use of molecular genetic approaches in assessing the parasite burden of wildlife and other little known species. There were various possible sources of error that could contribute to my lack of results, like malfunctions in the thermal cycler and spoiled PCR primers and lysis reagents. Some sources of failure leading to these results could be an incorrect lysis procedure for nematode tissue and incorrect primers for this particular worm species.

If this study was to be repeated, it would require more reagents to test, updated equipment, and adequate time to complete the troubleshooting necessary for positive results. Successfully identifying nematodes in species like the tapir could provide further insight into possible wildlife diseases that act as a contributing threat to wildlife and occasionally causing population declines. However, in order to justify the use of these

very meticulous molecular techniques, further research is needed regarding which methods are most effective to yield the best results.

CHAPTER TWO

Systematic Literature Review of the Success of Using Molecular Genetic Techniques to Describe the Parasite Community from Fecal Samples

Introduction

An ongoing debate revolving around the conservation of endangered wildlife species concerns the negative impact that direct, invasive handling for research purposes has on wildlife populations (Avise et al., 1979). In order to assess genetics of wildlife populations twenty plus years ago, it required the collection of fresh tissue in order to complete a protein electrophoresis, which often required the animal to be killed for scientific study (Proverbio, 2020). An alternative sample collection method that was introduced was blood/serum sampling to study serum proteins without having to kill the animal. When new genetic markers (restriction fragment length polymorphisms) were introduced, the sample collection process required destruction of the study specimen in order to extract mtDNA from fresh liver (Avise et al., 1979). The context of this argument changed with the development of the PCR (Saiki *et al.*, 1985).

PCR allows for the detection and reproduction of copious amounts of sequence-specific DNA and is used by researchers and clinicians to diagnose diseases, sequence genes, and complete quantitative and genomic studies in a rapid manner (Garibyan, 2014). PCR is an example of a molecular genetic procedure that can be completed with samples collected non-invasively from specimens.

Non-invasive techniques do not infiltrate nor destroy healthy tissue and do not involve tools that break skin or physically enter the body. These techniques can be seen

in animal conservation and medical research, but there are some cons to this type of sample collection. Although non-invasive collection methods are painless, they suffer from high levels of bacterial contamination, low gDNA production, or fragmented DNA strands (Mills et al., 2000).

According to current literature, morphological and molecular identification of nematodes are two growing methods in taxonomic and biodiversity studies. There has been a recent movement to stray away from the traditional methods of phenotypic identification due to the fact that these methods lack accuracy in identifying taxonomically challenging groups. In regards to molecular techniques, the data can violate the assumptions of phylogenetic analysis if sequences from various taxa are changing at different rates (Abebe et al., 2011). Despite the tangible advantages of molecular techniques, biologists and taxonomists worry that molecular techniques such as PCR could possibly replace taxonomy; reducing the complexity of an entire biological organism to a small fraction of that organism- the gene. The difficulty of linking DNA sequences to the ecological functions of whole species argues against abandonment of morphological identification all together (Tautz et al., 2002). Nevertheless the practical value of using molecular genetic methods for parasitological identification and quantification in wildlife is still in question because these are not model study species about which much is known. Based on this concern, and my unsuccessful experience using such an approach on the fecal parasites of Baird's tapir (Chapter One), I felt that a systematic review was needed to discover if using molecular genetic methods on fecal samples collected non-invasively are beneficial in wildlife parasitology, and to identify any challenges related to these methods.

I use a search of the scientific literature to find: a) which molecular approaches are most commonly used to identify parasites of wild animals, b) to describe geographic trends in samples sources, laboratory locations, and taxon inclusion, c) to evaluate whether researchers claimed that the molecular approach to parasitology was successful, and d) to identify the study characteristics associated with success.

Materials and Methods

A literature search was completed to find articles regarding the success rate of using molecular genetic techniques to identify, replicate, and sequence parasite DNA from fecal samples of wildlife, domestic animals, and humans.

A. Literature selection criteria

Several sources were searched in health-related search engines including ScienceDirect, PubMedCentral, and the University of Mississippi's One Search using the key words: nematode, helminth, molecular, non-invasive, and feces.

The search was limited to research articles and case studies/reports published in English from January 2008 to January 2020. First, the titles of the selected articles were examined to identify articles that reported on non-invasive fecal collection methods, helminths, and molecular methods such as PCR, electrophoresis, and DNA sequencing. If these search criteria could not be found in the title, the abstract was then examined. The data collected included the classification of the species being studied (i.e. domestic animal, captive animal, wild animal, or human), the sample size, the molecular methods used, and the success rate of the study completed. The successful articles were those that identified all of their studied helminths from their non-invasively collected samples, the partly successful articles only identified some of their helminths, and the unsuccessful studies did not identify any of their studied helminths. I excluded articles that did not include non-invasive sample collection methods, molecular methods, and helminth species. The literature selection process is demonstrated in **Fig 3.**

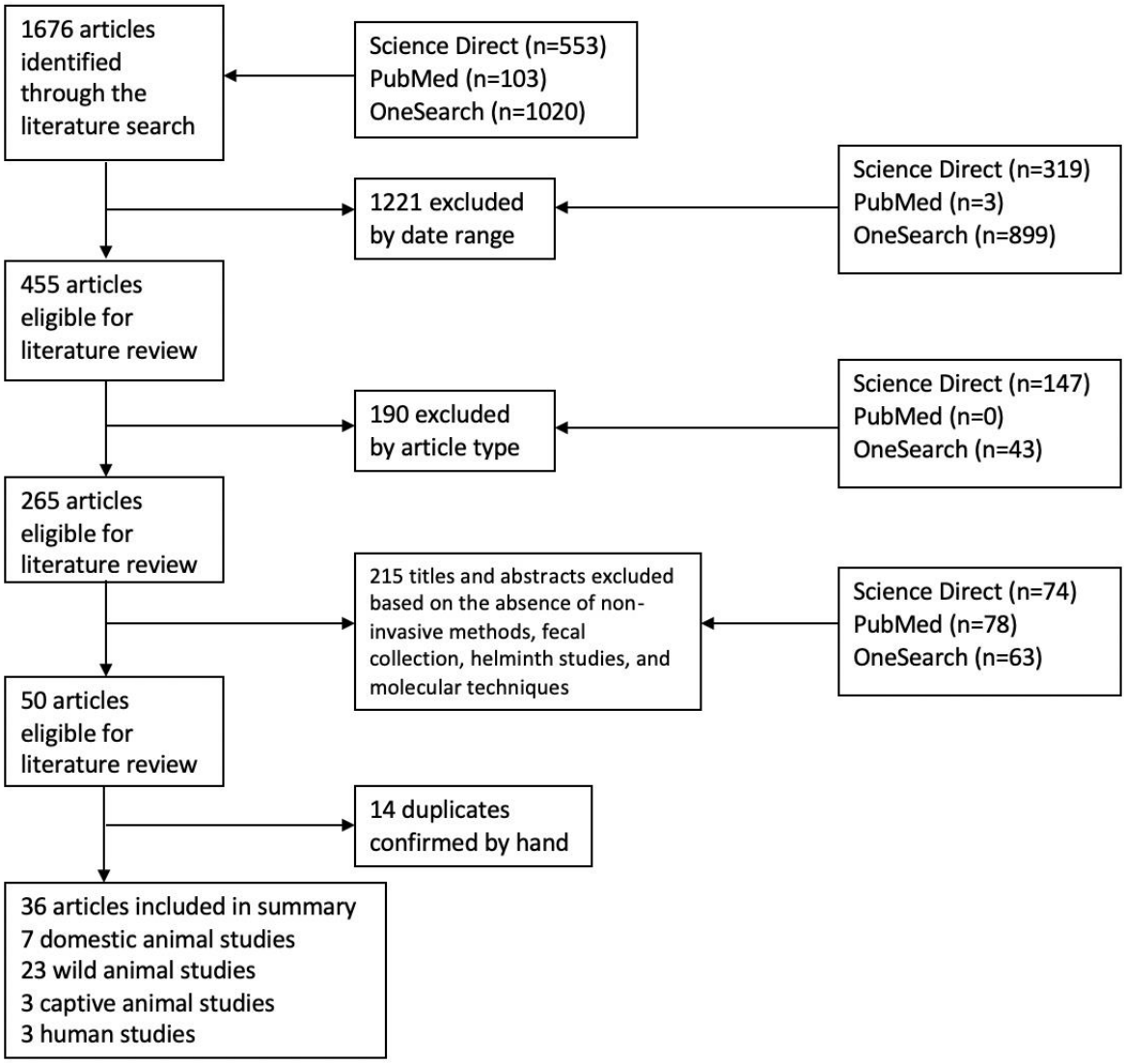


Fig 3. A flowchart showing the outcome for sample size of the decision process for identifying peer-reviewed, empirical literature suitable for providing data for this review.

Results

A. Initial literature search

A total of 1676 articles was identified in the initial literature search amongst all databases used. After excluding articles by date and article type, 265 were gathered. A screening of the title and abstracts was conducted, and 36 articles remained for this review.

B. Molecular methods/ sample sizes most commonly used

For sample collection, sample size was gathered from each article. The numbers of fecal samples that were collected and analyzed are as follows: eight articles (22.2%) reported 1-50 fecal samples, six (16.7%) articles reported 51-100 fecal samples, three (8.3%) articles reported 101-200 fecal samples, thirteen (36.1%) articles reported 200+ fecal samples, six (16.7%) articles did not report the sample size. It was also found that 9 (41%) of the 22 successful articles used 200+ fecal samples in their study, which was the fecal sample size most associated with success (**Fig 4)**. However, studies that sampled more than 100 fecal droppings were not more likely to yield partial or full success than studies examining fewer fecal samples (X^2 =1.0, df=1, p >0.05).In regards to the most common molecular methods used, thirty-six (100%) of the articles used PCR to replicate DNA. Two articles (5.56%) used only a traditional lysis method using proteinase K to extract DNA from their studied helminths. Fifteen articles (41.67%), on the other hand, performed lysis in addition to other methods to extract DNA. Ten articles (27.78%) used electrophoresis to separate DNA fragments (restriction fragment length polymorphism). Finally, twenty-eight of the articles (77.78%) sequenced the DNA.

C. Geographic trends and taxon inclusion

The articles that were selected were done in 18 countries (**Tables 3&4)** between the periods of 2008 to 2019. Three (8.33%) of the articles described human studies, twenty-four (66.67%) wild animal studies (**Fig 5)**, seven (19.44%) domestic animal studies (**Fig 6**), and 2 (5.56%) captive animal studies (**Fig 7**).

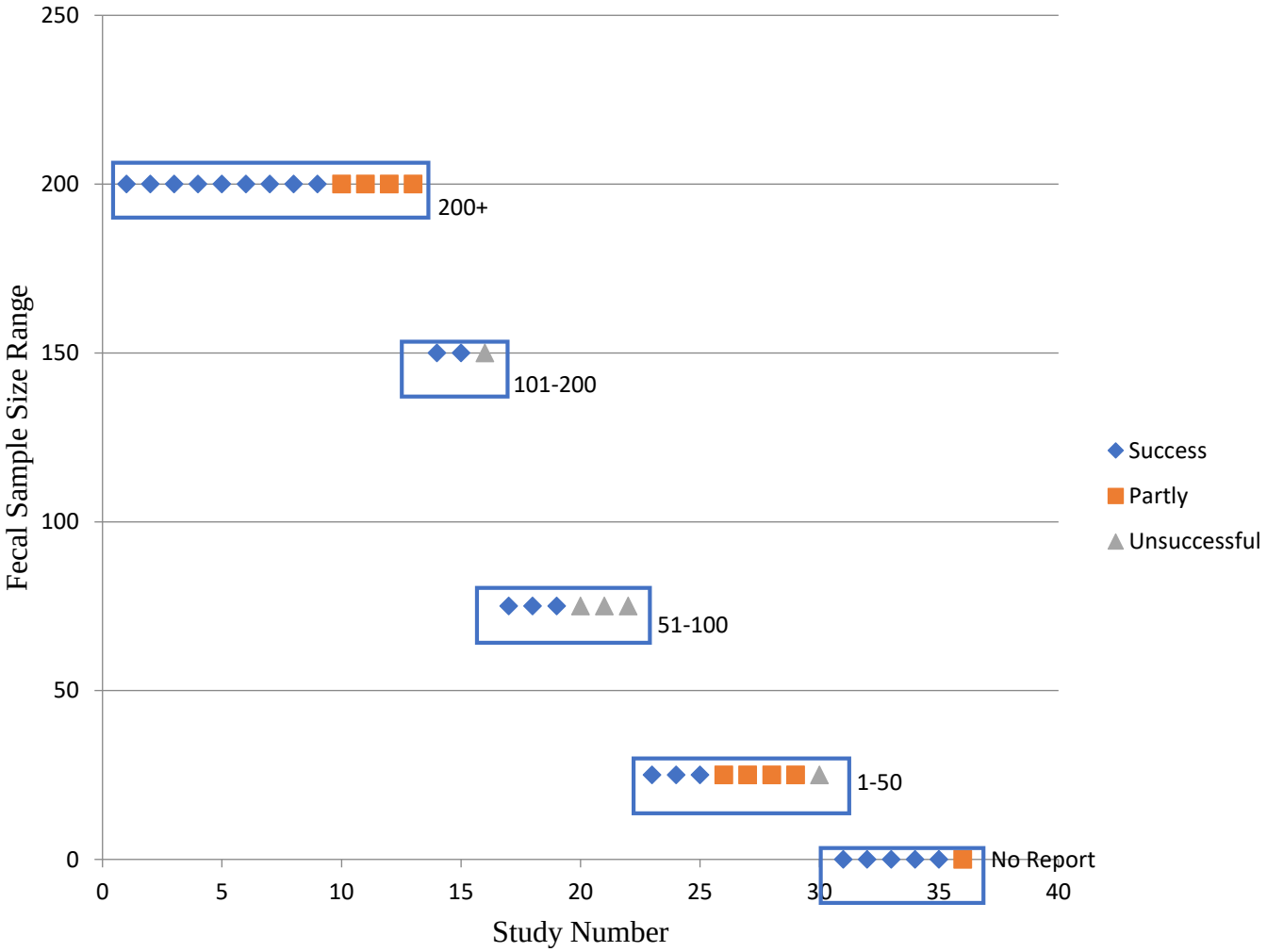


Fig 4. A graph that represents the fecal sample size most associated with successful study outcomes.

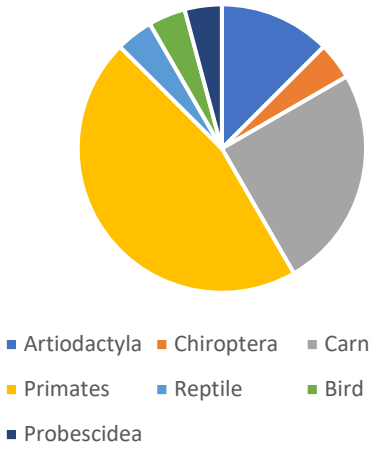


Fig 5. Percentage of each taxon group present in the wild animal studies.

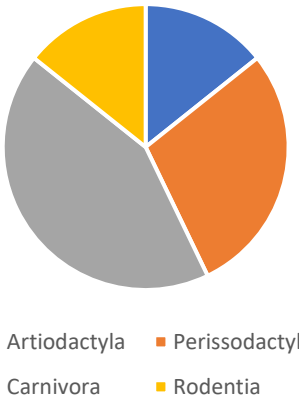


Fig 6. Percentage of each taxon group present in the domestic animal studies.

Fig 7. Percentage of each taxon group present in the captive animal studies.

Country	Amount of studies conducted in country
Uganda	5
Canada	3
Central African Republic	3
USA	3
Australia	2
Colombia	2
Germany	2
France	2
Mexico	2
Argentina	1
China	1
Germany	1
Greece	1
Kenya	1
Namibia	1
Rwanda	1
Tanzania	1
Zamibia	1

Table 3. The 18 countries covered in this review and how many studies were conducted in each.

Number of articles where the author was in the same country as the study species	21
Number of articles that had authors from areas outside the study species' country	12

Table 4. Authors' origins compared to the location of where lab work was completed.

D. Rate of success

The success rate of each article is as follows: 22 were successful in the molecular genetic identification of all of their studied nematodes, 3 were unsuccessful, 11 were partly successful in identifying only some nematodes in the study. The unsuccessful studies did not identify any of the nematodes in their study. Out of the successful studies, however, 18 already had sequences for their studied helminths named prior to their research.

Discussion

In this review, the most common molecular method used in wildlife parasitology was the polymerase chain reaction to replicate certain pieces of DNA that were successfully extracted from the nematode. Since this method was used in 100% of the articles, it can be concluded that the polymerase chain reaction, with successful DNA extraction, is a reliable source of replicating segments of DNA. Since the majority of the articles relied on sequences that were already added to the genetic library, determining the correct PCR primers and reagents was more simple compared to studies that did not have prior research on the nematodes present in their study species. For the nine studies that did not have a reference sequence prior to the study, more research was necessary in order to search databases for sequences of the closest relative to their study species. Then, primers had to be designed in order to sequence their DNA properly; proving to be a more painstaking and tedious process compared to studies that already had their reference sequences and recommended primers.

In regards to DNA extraction methods, only 5.56% of articles were successful in using the traditional lysis method (with proteinase K) to extract DNA from the studied helminths, while 41.67% of the articles used extraction methods such as phenol chloroform extraction, sodium hydroxide extraction, and physical disruption of the tissue followed up by PCR purification kits (i.e. SIGMA REDExtract-N-Amp Tissue PCR kit). This suggests that the sole use of the traditional lysis method using proteinase K to extract DNA from nematodes is not nearly as effective as using the other DNA extraction methods in addition to PCR purification kits. The articles that used the traditional lysis

method with proteinase K were mostly unsuccessful. For example, Vlčková et al. (2018) suggested that DNA degradation could have occurred due to incorrect storage, which led to their unsuccessful study.

The fecal sample size was also taken into consideration in this review. Most of the articles used 200+ fecal samples in their research, which was the highest number category of fecal samples studied. Prior to conducting any sort of lab or clinical study, sample size calculations should be considered in order to produce studies that are able to detect clinically pertinent differences (Altman, 1991). Using unnecessarily large sample sizes can waste resources and might pose ethical concerns if the samples are taken invasively. However, using small sample sizes may produce unreliable and irreproducible results (Faber, 2014). Since the 200+ sample size range was the range associated most with successful studies, it can be concluded that larger sample sizes in molecular genetic studies of animals would be recommended over smaller samples. Although none of the articles in this review specified on the waste produced by their use of 200+ fecal samples, they did specify that the techniques were all non-invasive, which takes away the ethical concerns of their study.

Out of all the taxon groups that were present in this systematic review, 66.67% conducted studies on wild animal species. The captive and domestic animal studies were used to discover nematodes that were possibly causing disease and stress within related wild animal species. For example, a study done by Lesniak (2017) focused on the identification of parasites in domestic dogs to help identify similar parasites causing the decline of wild grey wolves in that area. The other captive and domestic animal studies did not mention ecological concern in the study species, but focused on discovering if the

parasitic nematodes could be causing disease in the human population. The wild animal studies, on the other hand, had conservation objectives because parasitic nematodes can cause population decline of endangered species.

Because the largest loss of biodiversity is occurring in the tropics, more studies are necessary to identify parasites and other stressors related to this loss. Out of the 36 articles in this review, 21 of the articles were completed with studies in countries in, or on species from, the tropics.

Trends in geographical and institutional distribution of scientific research are very relevant in studying the ecosystem of the tropics. If research is limited to only a small number of countries, the science community's conclusion regarding the tropical ecosystems could be biased since these locations may not be broadly representative (Stocks, 2008). The majority of the articles in this review (60%) had authors that were from, and completing their lab work in, the same country as their study species. However, thirty-three percent of the studies collected samples from their study species and completed their lab work in countries outside of the area where their study species was found. Since a majority of the articles in the systematic review had authors that were from, and completing their lab work in the country of their study species, it can be concluded that the concern for biases is lower compared to the studies where the authors did not complete lab work in the region of their study species.

This systematic review revealed that 61.1% of the articles studied were successful in accomplishing their stated goal of non-invasive molecular identification of nematodes present in wildlife species and humans. However, 82% of the successful articles stated that they already had the sequences of their studied helminths in databases prior to their

study. For example, the study done by Solórzano-García (2017) focused on the non-invasive molecular techniques to identify parasites in howler and spider monkeys. Although the molecular methods could help with overcoming the limitations of traditional phenotypic identification, “its utility relies on the extant genetic library and the contributions that expand such library” (Solórzano-García, 2017).

Overall Conclusion

The two research projects of my thesis show that molecular genetic approaches to identifying and quantifying the parasite burden of animals using fecal sampling will be difficult and time-consuming but not impossible. For the foreseeable future experts on the morphological identification of nematodes and other helminths will remain essential to the development of reference DNA sequences for the wide variety parasitic taxa likely to be collected by wildlife biologists. Because morphological identification of individual parasitic worms is so time-consuming and labor intensive, however, manual counting based on morphology is not a practical means of monitoring disease stressors in threatened species given the immensity of the ongoing extinction crisis. New standard methodologies for sample collection, lysis and optimization and multiplexing of PCR must be adopted broadly in order to create reference sequence databases that can be used easily by a variety of types of biological scientists. The results of the systematic review of the literature suggest that progress is being made in capacity, building for molecular genetic approaches to parasitology in tropical countries.

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