



Identification of eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) infection levels in Northern bobwhite quail (*Colinus virginianus*) of the Rolling Plains, TX using a mobile research laboratory: Implications for regional surveillance

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ARTICLE INFO

Handled by: Jim Huggett

Keywords:

Bobwhite
Caecal
Eyeworm
Rolling plains
Surveillance
qPCR

ABSTRACT

Over the last few decades, there has been a decline in Northern bobwhite quail (*Colinus virginianus*) throughout their native range. While there are various factors that may be influencing this decline, it is suggested that parasites should be taken into consideration as a potential contributor in the Rolling Plains Ecoregion. High prevalence of the eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) in bobwhite of this region, coupled with a continuous decline, creates a need to assess infection through alternative methods for regional surveillance. Previous studies have developed a qPCR method and mobile research laboratory as an option for nonlethal procedures. However, there is still a need for standardization of these techniques. Therefore, this study builds on previous protocols to develop an application that considers factors that may influence qPCR results. In this study, cloacal swabs are collected from bobwhite in three locations throughout the Rolling Plains and scaled based on amount of feces present on the swab. This data is compared to qPCR standards as a limit of quantification for both eyeworm and caecal worm to define parasitic infection levels. Binary logistic regressions confirm that the probability of detection increases for both eyeworm (Odds Ratio: 2.3738; 95% Confidence Interval: [1.7804, 3.1649]) and caecal worm (Odds Ratio: 2.8516; 95% Confidence Interval: [2.2235, 3.6570]) as swab score increases. Infection levels for eyeworm and caecal worm are based on the generated cycle threshold value averages of qPCR standards. Based on the results of this study, this method can be applied in the mobile research laboratory to quantitatively assess regional parasitic infection in bobwhite throughout the Rolling Plains.

1. Introduction

Since the 1960s, there has been an annual decline of over 4% in Northern bobwhite quail (*Colinus virginianus* Linnaeus, 1758; hereafter bobwhite) throughout their native range [1]. This decline has received increasing attention in the Rolling Plains Ecoregion of West Texas, considered one of the last strongholds of this popular gamebird [2]. In this region, parasites have been an especially notable concern due to the prevalence of two heteroxenous nematodes, the eyeworm (*Oxyspirura petrowi* Skrjabin, 1929) and caecal worm (*Aulonocephalus pennula* Chandler, 1935), infecting bobwhite [3–6].

The wide-spread prevalence of the eyeworm and caecal worm in bobwhite of the Rolling Plains has led to a necessity for regional

surveillance. Common methods of assessing parasite prevalence in bobwhite have largely consisted of necropsies (e.g. [3,4,6,7]) which can involve time, money, and intense labor. Similar problems arise when using fecal floats as a nonlethal alternative, with the additional risk of potentially misidentifying parasite species' eggs [8]. Nonlethal procedures are made even more difficult given no reported evidence of eyeworm or caecal worm expulsion in the feces of bobwhite for morphological identification. Consequently, Kalyanasundaram et al. [9] successfully developed an accurate and precise multiplex quantitative PCR (qPCR) method to detect eyeworm and caecal worm egg presence in bobwhite feces, which has been utilized in a mobile research laboratory [10]. This progress has provided an opportunity to nonlethally assess parasitic infection in bobwhite and also provides a platform for

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<https://doi.org/10.1016/j.bdq.2019.100092>

Received 28 February 2019; Received in revised form 3 June 2019; Accepted 18 June 2019

Available online 05 September 2019

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widespread parasite surveillance in bobwhite populations throughout the Rolling Plains Ecoregion.

Regular surveillance of parasitic infection is vital as it can track the efficiency of mitigation efforts after rounds of treatment [11,12]. Additionally, surveillance may be especially important when considering that these nematodes are thought to enter diapause, a state of halted growth and development, when environmental conditions are poor [13]. Diapause has even been suggested to hinder the effectiveness of anthelmintic treatments due to parasitic inactivity at this time [14,15]. To prevent this, Easton et al. [16] suggests that qPCR assays may be useful in monitoring the intensity of parasitic infections in areas where multiple treatments have occurred to trace re-infections or low intensity infections.

While feces is the best sample type when using qPCR to detect eyeworms and caecal worms, it has been suggested that cloacal swabs are more efficient to collect in field applications [9,10,17]. However, cloacal swab efficiency in determining infection may be dependent on the amount of feces present in the swab and thus, may affect the outcome of qPCR results. This issue presents a need to standardize techniques that can be effectively communicated to landowners, hunters, and others in the Rolling Plains who may be interested in mitigation opportunities. Therefore, this study expands on the protocols outlined in Blanchard et al. [10], where cloacal swabs will be scaled based on the presence of feces on the swab in order to identify if scaling is an efficient method for determining infection. This study also examines appropriate ways for identifying low, moderate, and high infection based on cycle threshold (C_t) values in qPCR. Lastly, this study will also present a method for handling non-detects for eyeworm and caecal worm qPCR detection.

2. Materials and Methods

2.1. Ethics statement

This experiment was approved by Texas Tech University Animal Care and Use Committee under protocol 16071-08. All bobwhites were trapped and handled according to Texas Parks and Wildlife permit SPR-0715-095.

2.2. Sample collection and processing

The mobile research laboratory was deployed to three locations in the Rolling Plains Ecoregion including Cottle, Garza, and Mitchell Counties. Bobwhite sample collection occurred between March and October of 2018 following the procedures described in Dunham et al. [7] with an additional trap check in the afternoon for a total of 3 trap checks per day. Upon collection, bobwhites were banded, aged, sexed, weighed, given a body condition score, and a cloacal swab was taken. Birds that were collected and transported to The Institute of Environmental and Human Health Aviary were re-swabbed and had feces collected within 1–3 days of their arrival. Samples were processed in the mobile laboratory as described by Blanchard et al. [10]. Samples that were not immediately extracted upon collection were stored at -20°C until extraction.

2.3. DNA extraction and qPCR

DNA extraction protocols followed procedures outlined in Kalyanasundaram et al. [9] and Kistler et al. [17]. Fecal samples were weighed to 180–220 mg prior to extraction. Every 12th sample in a DNA extraction was used as a control for contamination. Quantitative PCR protocols used Taqman Fast Advanced Mastermix (Applied Biosystems, 4,444,557) with a final mastermix volume of 20 μL as described in Kalyanasundaram et al. [9] following the same qPCR running conditions. All samples were run in duplicates. Primer and probe sequences used (Sigma Aldrich, USA) in this study are presented in

Table 1. A negative control was used in each qPCR run on a 96-well plate (Applied Biosystems, 4,346,907). There was no evidence of contamination in the DNA extraction controls and qPCR controls.

2.4. Swab scoring

Swabs were scaled between 0–4 at the time of extraction. Fecal samples were also included in statistical analyses and denoted with a score of 5. The cloacal swab scaling is as follows: a 0 is scored when there is no visible fecal matter on the swab; a 1 has small flecks of feces on the swab to indicate the presence of feces; a 2 has small clumps of feces on the swab; a 3 has smears and clumps of fecal matter on the swab; and a 4 was used when excessively large clumps of feces were present and the swab was saturated in fecal matter (Fig. 1).

2.5. Infection level classification

Infection levels were classified by comparing C_t values generated for swabs to C_t values generated by the standards as a limit of quantification (LOQ). Standards in this study consist of serially diluted plasmid DNA collected from eyeworm and caecal worm and are the same standards outlined in Kalyanasundaram et al. [9] and Kistler et al. [17]. Concentrations for the standards ranged from 10^1 to 10^5 copy numbers.

2.6. Data analysis

Statistical applications were run in Minitab (v18). A total of 234 samples each for eyeworm and caecal worm were used in binary logistic regression analyses for the swab scoring data analysis. Probability distributions for the predicted probability of detection were generated from the binary logistic regression analyses.

3. Results and Discussion

Scoring as a method of health assessment has risen in popularity over the past several decades. This has been practiced in both terrestrial species [18,19] and marine mammals [20]. Swab scoring was adopted based on this concept in order to determine the efficiency of scoring in relation to the probability of detection of eyeworm and caecal worm infection by qPCR. Here, it is demonstrated that as the swab score increases from 0 to 5, the probability of detection for both eyeworm (Odds Ratio: 2.3738; 95% Confidence Interval: [1.7804, 3.1649]; Fig. 2A) and caecal worm (Odds Ratio: 2.8516; 95% Confidence Interval: [2.2235, 3.6570]; Fig. 2B) also increases. These statistical analyses confirm earlier conclusions of feces samples as the best method for identifying parasitic infection in bobwhite by qPCR [9,17]. While fecal samples have a higher likelihood of producing positive results, cloacal swabs are easier to collect in the field and reduce stress on the bird [17]. For this reason, when cloacal swabs are collected in the field, scores of 3 and 4 will be considered as more reliable data to represent infection in a population.

However, LOQs in qPCR should also be taken into consideration in determining cut-off values for positive results as well. Determining the LOQ should rely on DNA of a known concentration, like qPCR standards [21]. Therefore, the C_t value average of the lowest detected copy numbers of qPCR standards for each parasite should be considered as the LOQ, with any potentially positive result to be considered negative if above those C_t value averages. Based on this proposition and the standards represented in Fig. 3, infection levels for eyeworm are as follows: low infection is < 10 copy numbers, moderate infection at 10 copy numbers to 1,000 copy numbers, and high infection at $> 1,000$ copy numbers. For caecal worm, low infection is < 100 copy numbers, moderate infection at 100 copy numbers to 10,000 copy numbers, and high infection at $> 10,000$ copy numbers. These results are outlined with corresponding C_t value ranges in Table 2. In addition to the proposed LOQ and infection levels, previous cut-off values between a

Table 1
Primer and probe sequences used in multiplex qPCR of parasitic infection in bobwhite.

Primer/Probe	Sequence	Target	Product Size
Oxy2448F	GTTCCTCATGTGATTTCATTTTGT	Eyeworm ITS2 [17]	149 bp
Oxy2597R	ATAAACGTTATTGTTGCCATATGCT		
Oxy_Probe_1	FAM-AAAGAAAGGTAATTTCATCTGGT-MGB	Caecal Worm COX1 [9]	120 bp
Apen F1	GGGTTGTGGTACTAGGTGGGT		
Apen R1	GCACCCAAAATAGAACTCACCCC	Northern Bobwhite NADH2 [17]	79bp
Apen_Probe_1	VIC-GGTTCATCCTGGTAGAAGCGTTG-MGB		
ND2_70F	CAACCACTGAATCATAGCCTGAAC		
ND2_149R	GGTGGTGGGATTTTGAATGAG		
Quail_ND2_Probe1	NED-AGGAACCACAATCAC-MGB		

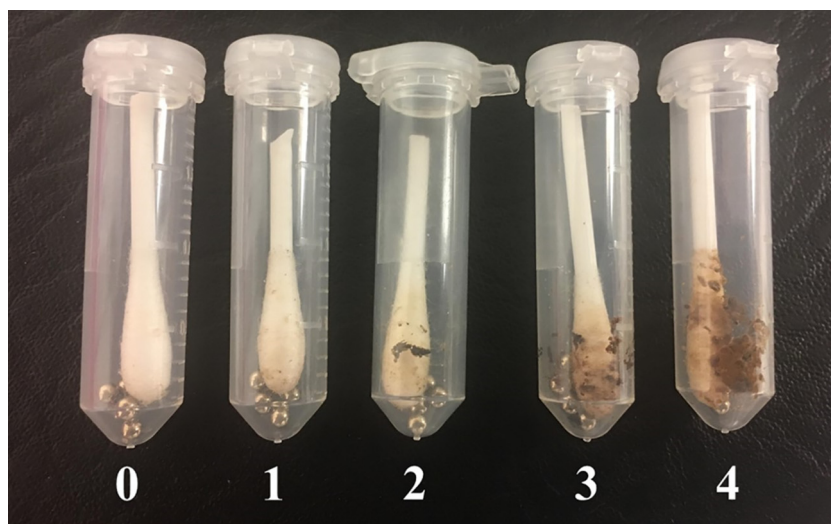


Fig. 1. Illustration of cloacal swab scores based on feces present on the swab.

positive and negative result for this qPCR method suggested in Blanchard et al. [10] should still be upheld.

Nevertheless, the issue remains that samples that are generated outside the threshold of detection in qPCR, or non-detects, need to be considered in order to appropriately assess overall infection regionally. There is much debate in how to treat these non-detects in quantitative analyses, with the aim to reduce bias where possible [22–24]. At present, there is not a singularly accepted method for handling non-detects in qPCR [25]. In order to maintain minimal bias in this study, it is proposed that non-detects could be substituted with the generated C_t value average of the lowest copy number. For example, based on values presented in this study, C_t value averages of ≥ 36 for caecal worm and ≥ 35.5 for eyeworm can be considered limits of quantification for either parasite and substitute non-detects. By substituting these values, an

overall infection level could be determined for a location by averaging all C_t values for that site. This method likely reduces bias because the limit of detection is based on quantifiable data. It may also reduce bias as McCall et al. [25] revealed that replacing non-detects with 35, instead of 0 or 40, reduced bias and suggested that non-detects may sometimes signify a failure to detect a true C_t value < 40 . Finally, the process of substituting non-detects becomes especially helpful with swab scores of 0–2 that generate non-detects but may not necessarily mean the bobwhite is uninfected. These individuals can be considered to have low infection to decrease the risk of a false negative, particularly when considering if other factors that facilitate infection spread may be present in the tested area.

Eyeworm and caecal worm infection levels of low, moderate, and high could also be used as an index of parasite prevalence in a

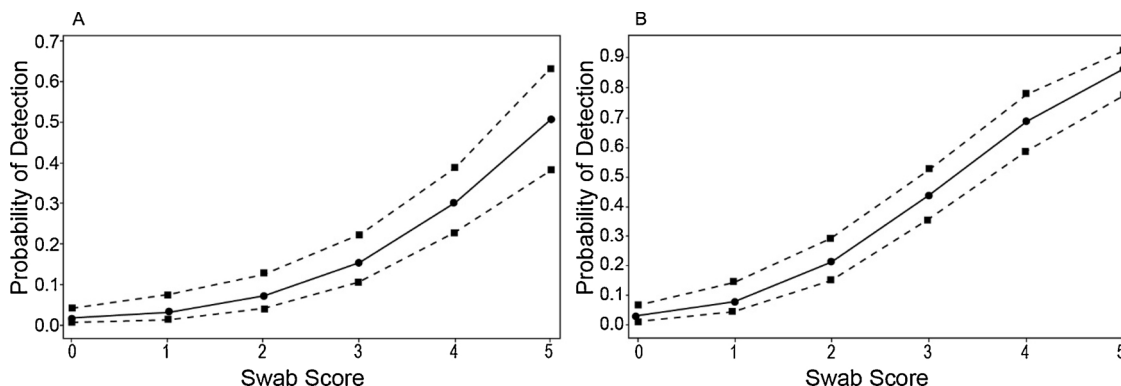


Fig. 2. Scatterplots of swab score and the predicted probability of detection of eyeworm (A) and caecal worm (B) by qPCR produced from binary logistic regressions. Solid lines depict estimated probability and dotted lines depict 95% confidence intervals.

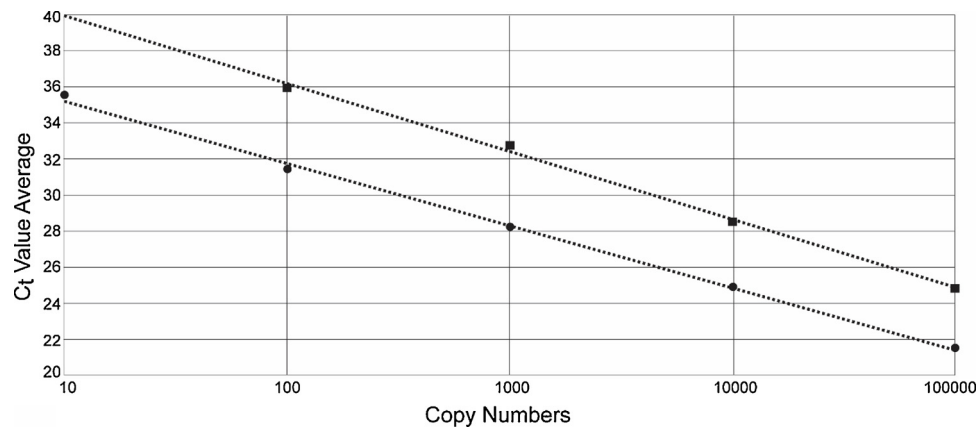


Fig. 3. qPCR standard curves for caecal worm (slope = -3.49, y-intercept = 42.65, Eff: 93.33%, $R^2 = 0.987$) as squares and eyeworm (slope = -3.46, y-intercept = 38.78, Eff: 94.54%, $R^2 = 0.997$) as circles, logarithmically transformed.

Table 2

Infection levels and corresponding copy number ranges and average C_t value ranges for eyeworm and caecal worm.

Infection Level	Eyeworm		Caecal Worm	
	Copy Number Range	Average C_t Value Range	Copy Number Range	Average C_t Value Range
Low	< 10	> 36	< 100	> 36
Moderate	10-1,000	28-36	100-10,000	29-36
High	> 1,000	< 28	> 10,000	< 29

population. Often times, these assessments can be combined with a variety of factors to comprise the overall health of a population. The index of biological integrity (IBI), for example, using fish assemblages is a popular method for regional assessments of aquatic environment health [26,27]. This index scores various physical, chemical, and biological conditions to rate water quality and aquatic communities. Similarly, in human medicine, the Braden scale is used often for accurately predicting the likelihood of elderly patients developing pressure ulcers based on scored evaluations [28]. A similar type of index assessment could be applied to bobwhite in the Rolling Plains. This could include a collaborative effort of analyzing and scoring factors like insect intermediate host diversity, habitat quality, eyeworm and caecal worm infection levels in the mobile laboratory, and nesting success to assess bobwhite population health in the Rolling Plains Ecoregion.

4. Conclusions

Overall, we conclude that assigning a scale to cloacal swabs collected in the field will help in accurately identifying eyeworm and caecal worm infection levels. Based on this data, scaling swabs in accordance with fecal material can aid in determining the probability of detection in qPCR, where higher swab scores lessen the likelihood of a false negative in bobwhite. These methods also provide a quantifiable estimate using the established LOQ to properly assess and compare infection levels across the Rolling Plains Ecoregion in addition to a level of infection that can be clearly communicated to stakeholders during the judgement and recommendation of mitigation procedures. Furthermore, with the application of these methods, the mobile research laboratory can perform consistent surveillance in areas receiving regular anthelmintic treatment while also comparing levels of infection regionally in relation to spatial and temporal factors that may influence eyeworm and caecal worm infection in bobwhite.

Funding

We thank Rolling Plains Quail Research Foundation (23A470) and

Park Cities Quail Coalition (23A540) for their financial support of our quail research.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest in this study.

Acknowledgements

We thank our sponsors for providing the financial means to make this study possible. We also thank the employees and volunteers of Matador Wildlife Management Area and Dalby Ranch in aiding our research efforts where possible. We also thank each of our study sites for providing us accommodations to perform this study. Finally, we thank all the laboratory members for their field and laboratory assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bdq.2019.100092>.

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