Veterinary Parasitology 253 (2018) 65–70

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

Development of a multiplex quantitative PCR assay for eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) detection in Northern bobwhite quail (*Colinus virginianus*) of the Rolling Plains Ecoregion, Texas

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ARTICLE INFO

Keywords:
COX1
Caecal worm
Eyeworm
ITS2
Multiplex qPCR
Quail

ABSTRACT

The Northern bobwhite quail (*Colinus virginianus*) is an economically significant gamebird that has experienced a decline throughout the Rolling Plains ecoregion of Texas. Recent surveys of this area have revealed a high prevalence in eyeworm (*Oxyspirura petrowi*) and caecal worm (*Aulonocephalus pennula*) infection that may contribute to this decline. In order to further understand these parasites role in bobwhite populations, a time-, and cost-effective multiplex quantitative PCR (qPCR) assay was developed in this study to detect eyeworm and caecal worm infection through egg detection using the ITS2 and COX1 gene region, respectively. Method validation for the qPCR involved bobwhite fecal samples from the Rolling Plains as well as samples spiked with eyeworm, caecal worm, and bobwhite DNA. Results showed an observed increasing qPCR parasite egg detection with increasing worm burdens. Future uses with this assay can also provide insight to seasonal parasite infection and the life cycles of eyeworm and caecal worm.

1. Introduction

Northern bobwhite quail (*Colinus virginianus*; hereafter bobwhite) have experienced a significant decline throughout the Rolling Plains ecoregion of Texas. This is alarming, as quail are of great economic significance to local communities of Texas as well as being a highly popular gamebird (Johnson et al., 2012). This regression continues to perplex researchers and hunters alike as habitat conditions in the Rolling Plains has remained relatively stable for the past decade (Rollins, 2007). Without any additional reasons behind this phenomenon, the investigation of parasites as a potential contributor has increased over the past several years (Dunham et al., 2014; Bruno, 2014; Dunham et al., 2016, 2017a,b).

Recent surveys of the Rolling Plains ecoregion have revealed an eyeworm (*Oxyspirura petrowi*) infection in bobwhite (Bruno, 2014; Dunham et al., 2016). The eyeworm, a heteroxenous nematode, has been reported to cause lesions in the harderian gland, cornea, and intraorbital glands (Dunham et al., 2016). Dunham et al. (2016) also noted visible attachment to the lacrimal duct which resulted in an increased inflammatory response in and around quail eyes. Between these lesions and inflammation, this could potentially decrease visual acuity in quail and thus increase susceptibility to predators and reduce forage ability.

In addition to the eyeworm, recent surveys have also indicated the presence of a caecal worm (*Aulonocephalus pennula*) in bobwhite (Bruno, 2014; Dunham et al., 2016). Research-to-date has revealed a lack of digesta in the caecum of bobwhites infected with caecal worms (Dunham et al., 2017a,b). Additionally, studies on gastrointestinal parasites in birds reveal extensive research regarding their role in weight loss, protein deficiency, and decreased immune responses (Clench and Mathias, 1995; Booth et al., 1993; O'Lorcain and Holland et al., 2000). Lastly, a recent phylogenetic study also indicated the caecal worm's 90% genetic relation to the family Ascarididae. Because of this close relation, it is possible that the caecal worm may have similar effects on its host as other members of the Ascarididae (Kalyanasundaram et al., 2017).

The role of parasites is often undervalued, despite evidence in their ability to regulate wildlife populations (Lehmann, 1984). Research by Dunham et al. (2016) showed that > 90% of the bobwhite sampled in their study were infected with *A. pennula* and *O. petrowi*. Such a prevalent infection requires a need to understand various aspects of these parasites including reproductive habits, seasonal infections, and the

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https://doi.org/10.1016/j.vetpar.2018.02.031
Received 29 June 2017; Received in revised form 14 February 2018; Accepted 17 February 2018

role of their intermediate and definitive host. However, current techniques often include necropsies for worm counts that require killing the bird or fecal float and McMaster slide methods for egg counts that are time-consuming and sometimes unreliable. Fecal float methods, in particular can result in misidentification of different parasite species' eggs (Zajac and Conboy, 2012). Therefore, a more advanced technique to accurately and efficiently detect parasite activity in bobwhites is necessary in order to better understand their potential impact.

As technology continues to advance, multiplex PCR has emerged as a successful application in diagnostic research (Elnifro et al., 2000). Since its introduction, multiplex PCR has paved the way for advances in cancer, parasitological, and disease research (Bernard and Wittwer, 2002; Elnifro et al., 2000). As a highly cost-effective method, multiplex PCR targets more than one sequence to be amplified at a time in a reaction (Stark et al., 2011). More importantly, detection time decreases substantially, allowing for quick and effective treatment, even before microscopy methods have been completed (Verweij et al., 2004).

In a previous study by Kistler et al. (2016), a duplex quantitative PCR (qPCR) protocol was developed and validated to identify eyeworm infection in bobwhite. The aim of this study is to develop a highly specific multiplex qPCR application to simultaneously target both eyeworm and caecal worm in bobwhite.

2. Materials and methods

2.1. Ethics statement

All quail were collected according to Texas Parks and Wildlife permits SRP-0715-095 and SRP-1098-984, and approved by Texas Tech University Animal Care and Use Committee protocols 16071-08 and 16049-06.

2.2. Study area and sample collection

The experimental study area of the present manuscript is consistent with the study area described in Dunham et al. (2014).Wild bobwhites were collected from the same study area, in the same manner, and using the same techniques as previously described by Dunham et al. (2014). The quail collection for the present study occurred in April 2017. Adults of caecal worm were collected from the caecum of bobwhites. All nematodes were washed repeatedly with 1X PBS. Male and female species were identified by morphological characteristics as described in Kalyanasundaram et al. (2017). Samples were preserved in 100% ethanol and stored at room temperature until DNA extraction.

2.3. Primer and probe design

Primers for eyeworm were previously designed by Kistler et al. (2016) and used for development of this multiplex qPCR. In this study, the sequenced COX1 gene region from Kalyanasundaram et al. (2017) was used to generate primers for the caecal worm. The forward and reverse primers for this study were designed using IDT PrimerQuest (http://www.idtdna.com/PrimerQuest/Home/Index). Hydrolysis probes were designed alongside caecal worm primers using the same COX1 sequence. While the ND2 (NADH dehydrogenase 2) sequence of bobwhite was used in this study, the reporter dye was changed to NED to maintain use of the bobwhite internal control. Probe sequences, reporter dyes, and quencher dyes are shown in Table 1.

2.4. Primer optimization

Purified genomic caecal worm DNA was tested through PCR with an annealing temperature gradient between 55 °C to 65 °C. Temperature gradient PCR reactions were run at 10 µl volumes containing 5 µl of 2X Red Dye Master mix (Bioline, USA), 0.5 µl of forward and reverse caecal worm primers, 3.0 µl of molecular grade water, and 1 µl of template caecal worm DNA. A negative control was run with each reaction using 1 µl molecular grade water in place of template DNA. Temperature gradient PCRs were run under the following parameters: 95 °C for 3 min; 29 cycles of 95 °C for 30 s, 55 °C–65 °C for 30 s, 70 °C for 15 s; and 70 °C for 5 min. All temperature gradient PCRs were run on a T100 Thermocycler (BioRad, USA). Amplification of the 120 bp product was visualized using a 2% agarose gel loaded with 5 µl PCR product. Each amplification was run using 100 bp DNA ladder (Fermentas, Thermo Scientific) to measure base pair fragments. Caecal worm primers were then tested at different concentrations including 25 µM, 50 µM and 100 µM alongside eyeworm and bobwhite primers to determine optimal concentration for the multiplex qPCR.

2.5. DNA extraction

Genomic DNA for primer optimization was extracted from adult caecal worms using DNeasy Blood & Tissue DNA Extraction Kit (Qiagen, USA) according to manufacturer instructions with one deviation of a final elution step of 50 µl molecular-grade water.

2.6. Standard optimization

Standards for eyeworm were designed following parameters described by Kistler et al. (2016). To prepare standards for caecal worm, caecal worm PCR products were amplified and purified using a PCR Purification Kit (Sigma Aldrich, USA). Ligation was done with a COX1 gene and pDrive vector according to manufacturer's instruction (Qiagen, USA) and transformed in QIAGEN EZ competent Cells (Qiagen). Positive clones were confirmed by colony PCR using M13 universal and caecal worm primers. Recombinant plasmids were purified using a Plasmid Purification Kit (Qiagen, USA) and sequenced using an automatic sequencer (Thermo Fisher Scientific). Purified plasmids were quantified using QUBIT 3.0 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Copy numbers of the plasmid were calculated based on the concentration and size of the recombinant plasmid. Plasmids were then serially diluted from 1×10^5 to 1×10^1.

All standards were optimized in qPCR reactions to generate a standard curve. Standards concentrations used ranged from 10^5 to 10^1. All sample DNA and standards were run as duplicates on a StepOnePlus real time PCR machine (Thermo Fisher Scientific) and results evaluated using StepOnePlus Software v2.3 (Thermo Fisher Scientific). Individual and multiplex qPCR reactions were run with the following parameters: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 20 µl reaction contained 10 µl of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), 0.04 µl of 100 µM eyeworm primers and probe, 0.02 µl of 100 µM bobwhite primers and probe, 0.02 µl of 25 µM caecal worm primers, 0.02 µl of 100 µM caecal worm probe, 0.1 µl of 10% BSA, 7.66 µl of molecular grade water and 2 µl of fecal DNA was used as a PCR template.

2.7. Multiplex qPCR method validation

Fecal samples were collected from the same 22 bobwhite for multiplex PCR method validation. All the collected fecal samples were stored at −20 °C until DNA extraction. DNA was extracted for qPCR validation using methods described by Kistler et al. (2016) with one deviation to final elution step of 50 µl molecular-grade water instead of 200 µl AE buffer. This deviation allowed optimal DNA concentration for parasite detection in multiplex qPCR. DNA was also extracted for qPCR validation from bobwhite breast tissue using DNeasy Blood & Tissue DNA Extraction Kit following manufacturer guidelines.

To validate the multiplex qPCR, 1 µl eyeworm DNA, 1 µl of caecal worm DNA, and 1 µl of bobwhite breast tissue DNA were added to wells not containing fecal DNA. This addition of parasite and quail DNA was used to determine detection efficiency of the qPCR tests. All the DNA samples were run in duplicates for eyeworm, caecal worm and