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Research paper

PCR detection of *Heterakis gallinarum* in environmental samples

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ABSTRACT

Heterakis gallinarum is a widely distributed cecal nematode that parasitizes gallinaceous birds including chickens and turkeys. *H. gallinarum* infection poses a problem for the poultry industry as the nematode egg serves as a vector for the protozoan parasite, *Histomonas meleagridis*, the causative agent of histomonosis. The only means of detecting *H. gallinarum* in the environment is microscopic identification of the eggs in soil or feces; however, *H. gallinarum* eggs are often mistaken for those of *Ascaridia galli*. Three primer sets were designed from sequences cloned from the *H. gallinarum* genome to develop a diagnostic PCR. Each of these primer sets amplified a single product from *H. gallinarum*, but were unable to amplify DNA from *H. meleagridis*, *Ascaridia galli*, or *Cestode* sp. *H. gallinarum* DNA was amplified from *Lumbricus* sp. (earthworms) and *Alphitobius diaperinus* (darkling beetles), confirming that the earthworm acts as a paratenic host for *H. gallinarum* and suggesting that the darkling beetle may be a carrier for this nematode.

1. Introduction

Heterakis gallinarum is a cecal nematode that infects many gallinaceous birds including chickens and turkeys (Lund and Chute, 1974), but it usually goes undetected in backyard and commercial flocks (Tyzzer, 1934; Kaushik and Sharma Deorani, 1969). Similar to other gastrointestinal nematodes common to domestic poultry, *H. gallinarum* infections have been associated with reduced weight gain (Daş et al., 2011). However, *H. gallinarum* is the vector for *Histomonas meleagridis* (Tyzzer and Fabyan, 1920; Tyzzer, 1926, 1934), a protozoan parasite that causes histomonosis (also known as blackhead disease) in gallinaceous birds and is also associated with reduced weight gain in chickens (Smith, 1895; Sigmon et al., 2019).

The earthworm is the only known paratenic host for *H. gallinarum*. Lund et al. (1966) demonstrated that earthworms infected with *H. gallinarum* larvae are capable of transmitting the nematode and *H. meleagridis* to turkeys and chickens. This observation may explain why some outbreaks of histomonosis in the poultry industry occur after heavy rains. However, no studies have been conducted to determine if *H. gallinarum* eggs are present in the soil or if earthworms are infected with the larvae on poultry facilities that experience repeated outbreaks.

Development of a highly sensitive polymerase chain reaction (PCR) that is specific to *H. gallinarum* DNA may be used to detect *H. gallinarum* in environmental samples. This type of molecular diagnostic tool would enable producers to identify areas of a poultry facility that are contaminated with the nematode egg or to investigate potential vectors of the nematode. The objective of this research was to design primer sets from sequenced fragments of the *H. gallinarum* genome that were: specific to *H. gallinarum*, conserved between *H. gallinarum* populations, and capable of amplifying *H. gallinarum* sequence from environmental DNA samples.

2. Materials and methods

2.1. Specimen collection

Mature *H. gallinarum* were collected from the ceca of chickens sampled from 6 different commercial and research flocks (2 flocks in Raleigh, NC; 1 flock in Kinston, NC; and 3 flocks from a processing plant in Marietta, GA. Mature *A. galli* and *Cestode* sp. were collected from 1 of the chicken flocks sampled in Raleigh, NC. *H. meleagridis* were isolated from a chicken sampled from a flock in Buford, GA. Seven earthworms were collected from a backyard chicken flock in Raleigh, NC, and 5 earthworms were collected from a commercial turkey facility in Clinton, NC. Darkling beetles (*Alphitobius diaperinus*) were collected from the North Carolina State University Talley Turkey Education Unit.

2.2. Experimental infection of darkling beetles with H. gallinarum eggs

Ten darkling beetles were placed in a petri dish for 1 week with 500 mg of turkey feed containing approximately 350 embryonated *H. gallinarum* eggs. The 5 beetles that survived to the end of the week were

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euthanized and placed into individual microcentrifuge tubes. The 5 beetles that died during the week were collected into a single microcentrifuge tube.

2.3. Genomic DNA isolation, DNA cloning and primer design

Genomic DNA (gDNA) from the first *H. gallinarum* population sampled from Marietta, GA was isolated using the Quick-gDNA MiniPrep kit (Genesee Scientific, San Diego, CA). All other gDNA isolation was accomplished using the PureLink Genomic DNA kit (Thermo Fisher Scientific, Waltham, MA).

H. gallinarum gDNA isolated from the first Marietta, GA population and pBluescript plasmid were digested with FastDigest EcoRI and XbaI restriction endonucleases (Thermo Fisher Scientific, Waltham, MA) at 37 °C for 1 h. One hundred fifty nanograms of digested *H. gallinarum* gDNA and 50 ng of digested plasmid were incubated with T4 DNA Ligase (Thermo Fisher Scientific, Waltham, MA) at room temperature for 2 h. The ligation products were transformed into GC5 competent cells according to manufacturer's protocol (Genesee Scientific, San Diego, CA). Fifty-four white colonies were transferred from sample plates to separate test tubes containing LB-amp with 100 µg/ml ampicillin and incubated at 37 °C for 16 h. Plasmid DNA was isolated from each bacterial culture using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

Plasmid DNA from several distinct clones (HG 1–4) was sequenced with T7 and T3 primers *via* Sanger sequencing at the Georgia Genomics and Bioinformatics Core at the University of Georgia, Athens, GA. A secondary set of sequencing primers were designed to sequence the full length of clones HG2 and HG4 (Table 1). Sequence information was deposited in Genebank: HG1 (MK122633), HG2 (MK122634), HG3 (MK122635), and HG4 (MK844591). A nucleotide BLAST search was conducted for each sequence to identify those that shared homology to other sequences in the database. Primers were designed to detect 3 sequences from HG 1, HG2 and HG3 using the IDT PrimerQuest Tool (https://www.idtdna.com) (Table 1).

2.4. PCR protocol

PCR reactions were run in an Applied Biosystems Veriti Thermal Cycler. All PCR reactions containing *H. gallinarum*, *A. galli*, *Cestode* sp., *H. meleagridis*, or darkling beetle gDNA were prepared to a final volume of 10 µl. All PCR reactions containing earthworm gDNA were prepared to a final volume of 20 µl. All PCR reactions contained 1x Phire Reactions Buffer with 1.5 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA), 200 µM dNTP mixture (Genesee Scientific, San Diego, CA), 0.5 µM forward and reverse primers, and 1x Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). A gradient PCR was prepared with samples containing 10 ng of *H. gallinarum* gDNA isolated from chickens in Raleigh, NC. The thermal profile for the gradient PCR was as follows: initial denaturation step at 98 °C for 1 min; followed by 35 cycles of 98 °C for 5 s; 55, 56, 57, 58, 59, or 60 °C for 5 s; 72 °C for 5 s; and final elongation at 72 °C for 1 min. The thermal profile for all subsequent PCR tests was the same using 60 °C as the annealing temperature. PCR samples containing DNA isolated from *A. galli*, *Cestode* sp., *H. meleagridis* and 5 populations of *H. gallinarum* used 10 ng of gDNA. The diagnostic PCR samples prepared with DNA isolated from earthworms collected from the backyard chicken pen or darkling beetles used 100 ng of gDNA, and the diagnostic PCR samples prepared with DNA isolated from earthworms collected from a commercial turkey facility used 200 ng of gDNA. The PCR products of all PCR tests were separated on 1.5% agarose gels prepared with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MN) at 100 V for 45 min. The gels were viewed under a blue light to observe the amplicon bands in each PCR sample.

2.5. Cloning and sequencing of PCR amplicons

HG1, HG2, and HG3 PCR amplicons produced from the combined Marietta, GA populations were excised from an agarose gel and purified using the PureLink Quick Gel Extraction and PCR Purification Combo kit (Thermo Fisher Scientific, Waltham, MN) and ligated into the pJET1.2 blunt Cloning Vector (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's directions. Ligation products were transformed and plasmid DNA isolated as above. Plasmid containing amplicons from HG1, HG2, and HG3 were Sanger sequenced at the Genomic Sciences Laboratory at North Carolina State University, Raleigh, NC. The sequences of the inserts were compared to the sequences used to design the primers in A Plasmid Editor (http://jorgensen.biology.utah.edu/wayned/ape/).

2.6. H. gallinarum gDNA serial dilution HG3 PCR

Ten-fold serial dilution standards were prepared from an isolate of *H. gallinarum* gDNA collected from 1 of the Marietta, GA populations. A single PCR reaction containing 1 ng of *H. gallinarum* gDNA was prepared as a positive control, and a no-template control (NTC) was prepared with biomolecular grade water. Triplicate HG3 PCR reactions were prepared from each serial dilution containing: 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg of *H. gallinarum* gDNA to determine the sensitivity of the HG3 primer set.

Table 1
HG1, HG2, and HG3 primer set information.

Primer	Primer sequence	Guanine & Cytosine content (%)	Primer length (bp)	Amplicon length (bp)
18S T7.2	5'-CGAACGGCTCATTACAACAG-3'	50	20	–
18S T3.2	5'-GACGATATACACTTTGAGAGCTGG-3'	45.83	24	
HG2 T7.2	5'-CGTTGATGTTTGGTAGCCAC-3'	50	20	–
HG2 T3.2	5'-ACCATCTCCTTTGTACTTGACG-3'	45.45	22	
HG1 Forward	5'-GCGCCTGACTCTCGAATTTA-3'	50	20	118
HG1 Reverse	5'-ACTGTATTCCTTAACCGCACTC-3'	45.5	22	
HG2 Forward	5'-GACCGGCGAATTTAGCATATTG-3'	45.5	22	99
HG2 Reverse	5'-AGGAGGGCTGGTAACTATGTA-3'	47.6	21	
HG3 Forward	5'-GCATCTTGGCATGCGTTAAG-3'	50	20	138
HG3 Reverse	5'-ACGCTACCGTAGTCCATACT-3'	50	20	

3. Results

3.1. Sequence analysis of cloned H. gallinarum gDNA

Limited sequence information is available for *H. gallinarum* (Smythe et al., 2006; Jiménez et al., 2012; Wang et al., 2016; Gu et al., 2016, 2018). Fragments of the *H. gallinarum* genome were cloned into pBluescript vector and sequenced. Sequence from clone HG4 (MK844591) shared 100% sequence identity with 100% coverage of the partial 18S ribosomal RNA gene for *H. gallinarum* (DQ503462). Sequences from clones HG1 (MK122633), HG2 (MK122634), and HG3 (MK122635) were selected for designing primers (Table 1). No significant sequence identity was found in BLASTN (megablast) for all three sequences whereas 45% similarity covering 18% of an unknown protein sequence found in the nematode, *Anisakis simplex*, was observed for HG2 using BLASTX.

3.2. Primer specificity and sensitivity evaluation

A gradient PCR was conducted to test the amplification efficiency of each primer set at annealing temperatures between 55 °C and 60 °C to select an annealing temperature that would increase primer specificity without compromising binding affinity for the target sequences. All 3 primer sets amplified the target sequence at all tested annealing temperatures in the gradient PCR (Fig. 1). The fluorescence intensity for HG1 decreased mildly between 56 °C and 57 °C. No discernable variation in fluorescence intensity was observed between HG2 or HG3 samples. The fluorescence intensity in the HG3 bands did not vary between annealing temperatures. Sixty degrees Celsius was selected as the annealing temperature for all subsequent PCR tests.

The species specificity of the primer sets and conservation of the target sequences were tested by PCR amplification of gDNA isolates from single populations of *H. meleagridis*, *A. galli,* and *Cestode* sp. and 5 populations of *H. gallinarum* collected from chicken flocks from Raleigh, NC; Kinston, NC; and Marietta, GA. All 3 primer sets amplified a single amplicon in all 5 *H. gallinarum* isolates and were unable to amplify any sequence from the *H. meleagridis*, *A. galli*, or *Cestode* sp. samples (Fig. 2). The fluorescence intensities of the amplicon bands appeared equal between *H. gallinarum* samples for each primer set. Sequencing of HG1, HG2, and HG3 amplicons PCR purified from a mixed sample of gDNA from 2 Marietta, GA *H. gallinarum* populations showed all 3 amplicon sequences exactly matched the sequences used to design the primers

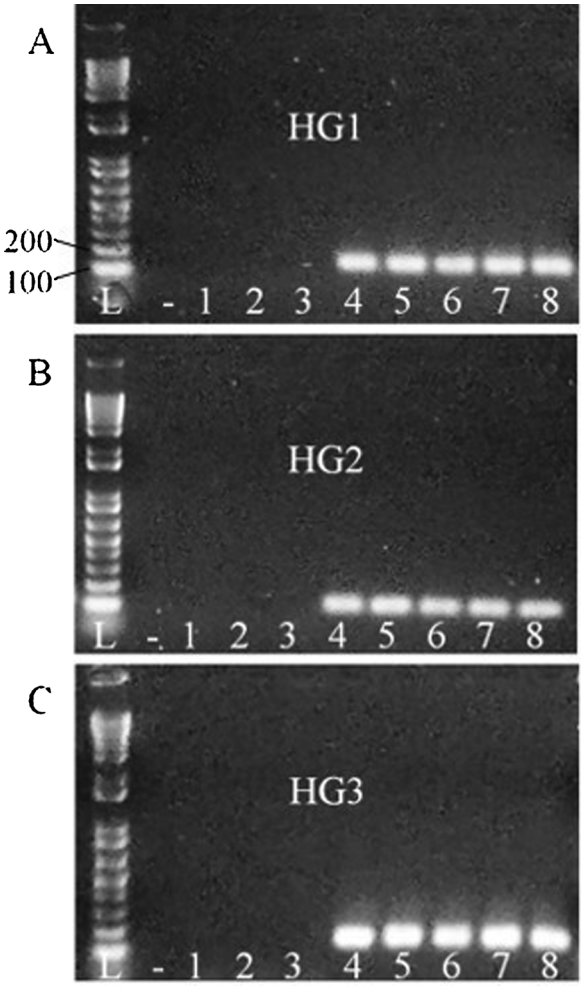

Fig. 2. Image of agarose gel containing PCR products of gDNA isolates from *H. meleagridis* collected from culture (1), *Cestode* sp. collected from a chicken (2), *A. galli* collected from a chicken (3), and 5 populations of *H. gallinarum* collected from distinct chicken flocks (4–8) amplified with A) HG1, B) HG2, and C) HG3 primer sets. L = 1 kb plus DNA Ladder.

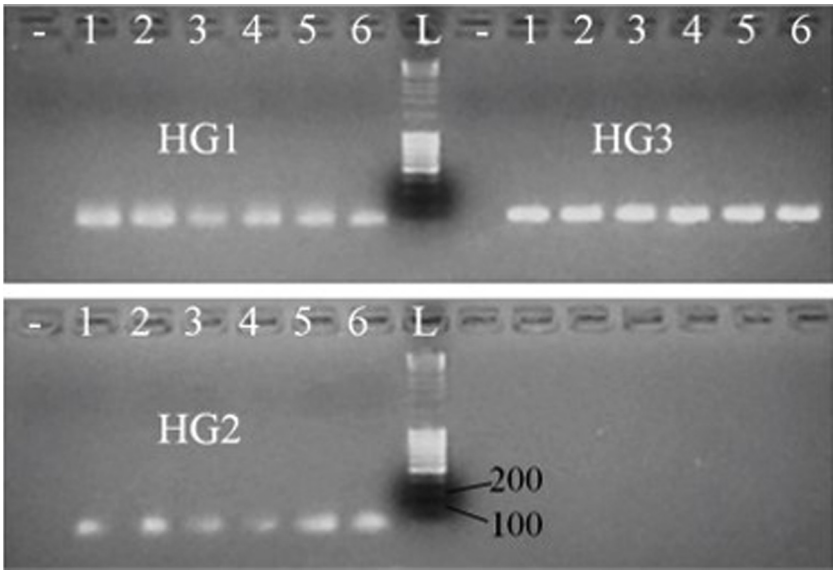

Fig. 1. Image of agarose gel containing gradient PCR products of HG1, HG2, and HG3 primer sets for each tested annealing temperature: 1 (55°C), 2 (56°C), 3 (57°C), 4 (58°C), 5 (59°C) and 6 (60 °C). L = 1 kb plus DNA Ladder (Thermo Fisher Scientific, Waltham, MN).

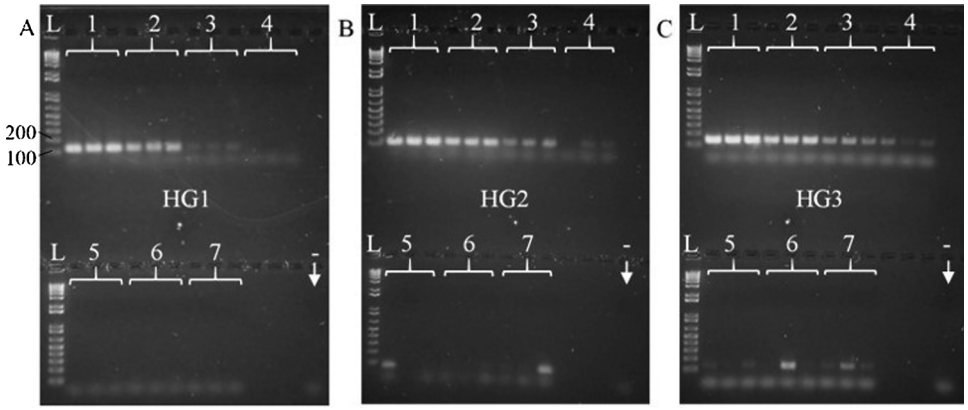

Fig. 3. Image of agarose gel containing PCR products of 5-fold *H. gallinarum* gDNA serial dilutions: 5 ng (1), 1 ng (2), 200 pg (3), 40 pg (4), 8 pg (5), 1.6 pg (6), 320 fg (7). L = 1 kb plus DNA Ladder.

(data not shown).

The sensitivity of the primer sets to target DNA sequences was evaluated by preparing a set of 5-fold serial dilution standards of *H. gallinarum* gDNA isolated from the chicken flock in Kinston, NC. Triplicate PCR reactions were prepared for each primer set containing 5 ng, 1 ng, 200 pg, 40 pg, 8 pg, 1.6 pg, and 320 fg of *H. gallinarum* gDNA. The reliable limit for detection of *H. gallinarum* gDNA was determined for each primer set based on the decline in consistent amplification of target in all 3 replicates of a given gDNA standard. HG1 primers amplified target in all 3 replicates of the 5 ng, 1 ng, and 200 pg samples, but failed to amplify target in any of the more dilute reactions (Fig. 3A). The florescence intensity of the amplicon bands steadily declined from the 5 ng samples to the 200 pg samples, but fluorescence intensity was consistent between replicates for these 3 gDNA standards. HG2 primers amplified target in all 3 replicates of the 5 ng, 1 ng, and 200 pg, but only amplified target in 2 of the 40 pg samples, 1 of the 8 pg samples, 1 of the 1.6 pg samples, and all 3 of the 320 fg samples (Fig. 3B). The fluorescence intensity of the amplicon bands steadily decreased from the 5 ng to the 200 pg samples, but successful amplification of target and the fluorescence intensity of amplicons in the more dilute samples was variable; all but 1 of the amplicon bands in the 1.6 pg and 320 fg samples were very faint. HG3 primers amplified target in all 3 replicates of the 5 ng, 1 ng, 200 pg, and 40 pg samples, but only amplified target in 2 of the *8 pg* samples, 2 of the 1.6 pg samples, and all 3 of the 320 fg samples (Fig. 3C). The fluorescence intensity of the amplicon bands steadily decreased from the 5 ng to the 40 pg samples. The fluorescence intensity of amplicons between replicates of each standard was consistent for the 5 ng, 1 ng, and 200 pg samples, but became more variable in the 40 pg samples. Fluorescence intensity of the amplicon bands was inconsistent between replicates of the 1.6 pg and 320 fg samples. The HG3 primer set demonstrated consistent amplification of *H. gallinarum* sequence at lower concentrations of gDNA template than HG1 or HG2 primer sets and was selected for testing the diagnostic PCR protocol on earthworm and darkling beetle environmental samples.

3.3. *H. gallinarum* detection in earthworms and darkling beetles

To assess the feasibility of using the *H. gallinarum* diagnostic PCR with environmental samples from poultry facilities, the diagnostic PCR protocol was tested with gDNA isolated from 7 earthworms that were collected from a backyard chicken flock. This flock was previously determined to be infected with *H. gallinarum* based on earlier coprologic examinations (data not shown). PCR amplification of a single band at approximately 138 bp was detected from Worms 1, 2, 3, 5, and 7 (Fig. 4).

To test whether *H. gallinarum* DNA could be amplified from earthworms isolated from a turkey facility experiencing histomonosis, gDNA

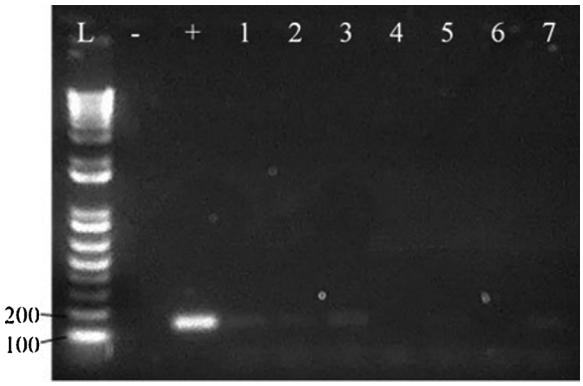

Fig. 4. Image of agarose gel containing products from a diagnostic PCR of gDNA isolates from 7 earthworms collected from a backyard chicken pen. Each number on the gel corresponds to a different earthworm. L = 1 kb plus DNA Ladder.

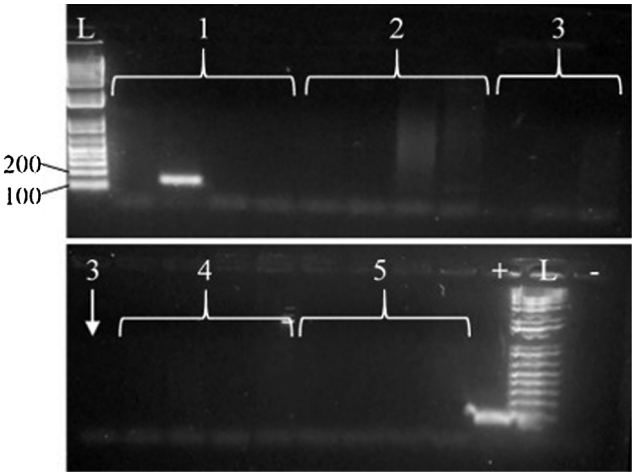

Fig. 5. Image of agarose gel containing products from a diagnostic PCR of the 4 pieces of 5 earthworms collected from a commercial turkey facility. Each number on the gel groups the 4 pieces collected from an individual earthworm. L = 1 kb plus DNA Ladder.

isolated from 5 earthworms collected from the property were subjected to PCR amplification by HG3 primers. The second isolate from Worm 1 and the fourth isolate from Worm 2 amplified HG3 target sequence (Fig. 5). All other worm isolates were negative.

Darkling beetles were tested as potential carriers of *H. gallinarum* in a diagnostic PCR with gDNA isolated from darkling beetles that had

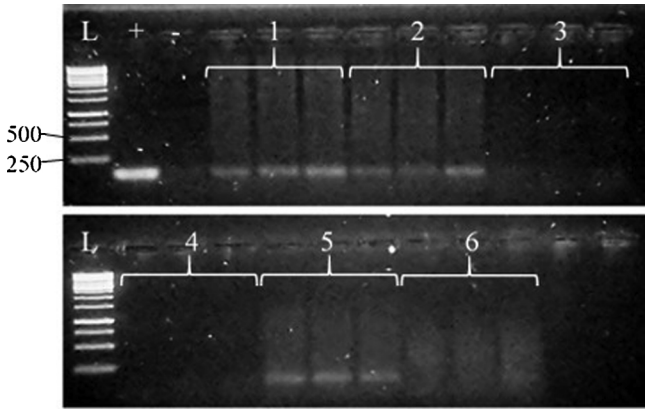

Fig. 6. Image of agarose gel containing products from a diagnostic PCR of gDNA isolates from darkling beetles 1 week after feeding them *H. gallinarum* eggs. The 3 replicate PCR reactions for each darkling beetle isolate are grouped under the corresponding DNA isolate: the 5 live darkling beetle isolates (1–5), and the isolate consisting of 5 dead darkling beetles (6). L = GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, MN).

been fed embryonated *H. gallinarum* eggs. No eggs were observed attached to the exterior of the darkling beetles prior to DNA isolation. Three samples prepared from live beetles (Beetle 1, 2, and 5) amplified the HG3 target (Fig. 6). All other samples, including the dead beetles, were negative.

4. Discussion

There is limited genomic information available for *H. gallinarum*, with only mitochondrial and ribosomal sequences known (Smythe et al., 2006; Jiménez et al., 2012; Wang et al., 2016; Gu et al., 2016, 2018). Designing primers against these conserved sequences often results in amplification that is not species-specific and requires sequencing of the amplicon for proper diagnosis (Ibáñez-Escribano et al., 2014; Boubaker et al., 2016). For instance, the 18S sequence for *H. gallinarum* has 98% sequence identity to the distantly related *A. galli*.

To generate sequence information, short fragments of the genome were cloned and sequenced from a population of *H. gallinarum*. The identification of a clone (HG4) containing the known *H. gallinarum* 18S sequence suggests that the other sequences used in this study are also *H. gallinarum*. This conclusion is also supported by the similarity of HG2 to an unknown protein in the nematode *A. simplex*. HG1, HG2, and HG3 were chosen to design primers against because each of these sequences had no significant similarity in GenBank as determined by BLASTN. Although the *H. gallinarum* 18S sequence was identified, there is no *H. gallinarum* reference genome to compare with the other sequences used in this study. Therefore, the specificity of the primers was tested using gDNA isolated from 5 different populations of *H. gallinarum* and other gastrointestinal parasites common to poultry including *H. meleagridis*, *Cestode* sp. and a distantly related species of nematode, *A. galli*. Genomic DNA samples from all 5 *H. gallinarum* populations produced an amplicon of the predicted size for each primer set while none of the primers amplified DNA from the *H. meleagridis*, *A. galli*, or *Cestode* sp. samples. The *H. meleagridis* used in the study was obtained from an *in vitro* culture containing *H. meleagridis* and cecal bacteria. Therefore, it is likely that the primer sets are unable to amplify product from bacteria either since no amplicons were produced in the PCR samples prepared with gDNA isolated from these cultures. Based on this sequence and PCR data, it may be determined that all 3 primer sets specifically amplify *H. gallinarum* sequences.

The sequences the primers anneal to must be conserved between populations of *H. gallinarum* to ensure the diagnostic PCR can identify *H. gallinarum* DNA in environmental samples collected from different populations of nematodes. Each primer set amplified target sequence from all *H. gallinarum* gDNA isolates tested in this study, suggesting that the target sequence of each primer set is conserved in the *H. gallinarum* genome. To assess the degree of sequence conservation of the amplified regions between *H. gallinarum* populations, HG1, HG2, and HG3 amplicons produced by amplification of a pooled sample of gDNA from the *H. gallinarum* populations collected from the Marietta, GA processing plant were cloned and sequenced. The sequences of these amplicons shared 100% identity with the sequences obtained from the original gDNA fragments, further indicating that the sequences of the genomic regions amplified by each primer set are highly conserved between distinct populations of *H. gallinarum*. Although the genetic diversity among *H. gallinarum* populations is low (Amor et al., 2018), it is possible populations of *H. gallinarum* not tested in this study possess mutations in 1 or more of the sequences amplified by these primer sets. Using 1 of the primer sets to test samples containing *H. gallinarum* from unknown populations may result in failed detection of *H. gallinarum* DNA. The use of a combination of these primer sets would further increase the accuracy of the diagnostic test for detecting *H. gallinarum*. For simplicity, only 1 primer set was used for testing the diagnostic PCR protocol on environmental samples in this study. The HG3 primer set was selected because it produced the lowest consistent threshold for detection of *H. gallinarum* gDNA, approximately 40 pg, compared to approximately 200 pg for HG1 and HG2.

Earthworms were selected as the first environmental samples to test the *H. gallinarum* diagnostic PCR for 2 reasons: *H. gallinarum* larvae have been shown to hatch inside the earthworm's intestines and burrow into the worm's tissues (Lund et al., 1966), and earthworms continually ingest *H. gallinarum* eggs from the soil throughout the spring and summer (Lund et al., 1966) thereby concentrating the unshelled larvae in their bodies. Backyard chickens are more prone to parasitic infections than commercial poultry because they have access to outdoor pen areas (Permin et al., 1999). Earthworms collected from a backyard chicken pen were used as the first PCR test subjects because previous microscopic examination of chicken feces and soil from the pen indicated the area was contaminated with *H. gallinarum* eggs. Of the 7 earthworms sampled, 5 were positive for the HG3 target sequence indicating the PCR protocol is capable of positively identifying *H. gallinarum* from environmental samples. While it was not the aim of this experiment to survey the species or proportion of earthworms that may be carrying *H. gallinarum*, based on these results, it appears that a large percentage of earthworms from backyard chicken flocks may be infected with *H. gallinarum* larvae, a demographic that may warrant further investigation.

Turkeys are a less suitable host for *H. gallinarum* than chickens (Lund et al., 1974) and generally harbor lower numbers of adult nematodes capable of producing embryonated eggs. However, *H. gallinarum* do not need to reach maturity to transmit *H. meleagridis* to their host. Chickens are capable of harboring both *H. gallinarum* and *H. meleagridis* in the absence of disease (Powell et al., 2009), and act as carriers producing large numbers of *H. meleagridis*-infected *H. gallinarum* eggs which can survive in the environment for 3 years (Farr, 1961). Earthworms were collected from a commercial turkey facility (which had been converted from a chicken facility 2 years earlier) during a histomonosis outbreak to investigate the possibility the earthworms ingested *H. gallinarum* eggs speculated to have been left behind by the chickens. Based on the results of the PCR test, it is possible the outbreak was initiated by ingestion of earthworms carrying *H. meleagridis*-infected *H. gallinarum*. Although the chickens formerly raised on the property were likely to be the source of the *H. gallinarum* eggs, this cannot be proven and goes beyond the scope of this research. The data presented for both diagnostic tests performed with earthworm gDNA isolates demonstrates that environmental contamination with *H. gallinarum* eggs may be diagnosed on a poultry facility by identifying *H. gallinarum* DNA from earthworms.

Darkling beetles have been speculated as vectors of *H. gallinarum* or *H. meleagridis*, but no studies have confirmed their role in the

transmission of either parasite. Huber and colleges identified *H. meleagridis* DNA *via* PCR in only a small percentage of darkling beetle mealworms from 1 chicken and 1 turkey facility experiencing histomonosis outbreaks (Huber et al., 2007). No data has been published demonstrating darkling beetles as carriers of *H. gallinarum* prior to the current study. Three of the live darkling beetles exposed to *H. gallinarum* eggs were positive for the HG3 target sequence which indicates darkling beetles are capable of transporting *H. gallinarum* eggs; however, this does not demonstrate whether the *H. gallinarum* larvae are alive and able to be transmitted by the darkling beetles. Although no *H. gallinarum* eggs were observed attached to the darkling beetles, it is still unclear whether the beetles mechanically transport or ingest the eggs. This data represents the first molecular evidence that darkling beetles may carry *H. gallinarum* and demonstrates the potential of using the *H. gallinarum* diagnostic PCR to investigate reservoirs and vectors for transmitting *H. gallinarum* between poultry facilities.

5. Conclusion

Three PCR primer sets were designed and tested for specificity to *H. gallinarum* and were found to be suitable for use in an *H. gallinarum* diagnostic PCR as the amplified sequences were specific to *H. gallinarum* and conserved between populations. Earthworms sampled from a backyard chicken pen and a commercial turkey facility were both found to carry *H. gallinarum* DNA which demonstrates the potential for using the diagnostic PCR to investigate sources of *H. gallinarum* on poultry facilities. *H. gallinarum* DNA was amplified from darkling beetles experimentally exposed to the nematode's eggs, indicating further research is needed to evaluate the potential of the darkling beetle to act as a vector or mechanical carrier for *H. gallinarum*. The PCR primers presented in this paper may be used as a research tool for identifying potential *H. gallinarum* vectors and investigating alternate modes of transmission of the nematode.

Acknowledgements

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