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A novel PCR assay and sampling techniques for the detection of *Raillietiella orientalis*

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Raillietiella orientalis, an invasive crustacean pentastome parasite, threatens native snake populations in the southeastern United States, infecting at least 18 species across Florida. Pentastome parasites have complex life cycles, with snakes often serving as definitive hosts for adult parasites that attach to the lungs and shed eggs into the host feces. PCR assays exist that distinguish invertebrate species via amplification and sequencing of mitochondrial DNA fragments. However, no molecular assays specific for R. orientalis or optimized fecal flotation methods for pentastome egg detection are available. We developed a novel PCR assay targeting the R. orientalis cytochrome c oxidase subunit I (CO1) gene and validated it across 611 samples, including cloacal swabs and fecal samples from live and deceased snakes, multiple pentastome species, and confirmed positive and negative control snakes diagnosed from lung dissections, morphology, and sequencing. We also compared the wet mount microscopy and three fecal flotation techniques for egg detection and assessed the impact of aging and drying on the fecal sample effectiveness. Our PCR assay demonstrated 100% specificity for R. orientalis across all sample types with 98% sensitivity for R. orientalis adults, larvae, and eggs (feces). The lowest sensitivity was observed in cloacal swabs (22%). The PCR assay was tested in a separate laboratory with similar results. Wet mount microscopy was more effective than fecal flotation for egg counts, though the false negative rate did not differ significantly between methods. Aging feces reduced egg counts but did not significantly increase the number of false negatives. Based on these results, we recommend using fecal samples from live snakes as the primary detection method, supplemented by cloacal swabs. These optimized methods are critical for improving surveillance of R. orientalis and characterizing the threat of this invasive pentastome to native snake species in the southeastern United States.

KEYWORDS

pentastome, reptiles, snake parasites, parasite detection, fecal sampling, emerging disease

Introduction

Emerging infectious diseases are major contributors to wildlife population declines, extirpation events, and extinctions of wildlife (Lorch et al., 2016; Cheng et al., 2021; Akçakaya et al., 2023; Puryear and Runstadler, 2024). Further, the anthropogenic pressures of development, globalization, and climate change may enhance the spread of pathogens, making emerging epidemics a constant challenge for wildlife and informed management action critical (Daszak et al., 2001; Tompkins et al., 2015; Luberto et al., 2024). This creates a consistent need for the development of reliable and cost-effective tools for detection of pathogens that are accessible to the research community as well as conservation practitioners. For example, several detection tools have been developed that are now essential for understanding infection dynamics of fungal pathogens on wildlife species globally. In particular, quantitative PCR assays specific to chytrid fungi (Batrachochytrium dendrobatidis and B. salamandrivorans) (Kerby et al., 2013), the keratinophilic fungus Ophidiomyces ophidiicola (Bohuski et al., 2015), and the psychrophilic fungus Pseudogymnoascus destructans (Muller et al., 2013) have been essential to characterizing fungal diseases in amphibian, snake, and bat populations, respectively. As novel pathogen threats continue to emerge, rapid development of robust detection tools is necessary for surveillance and understanding pathogen spread and epidemiology.

A recently described threat to herpetofauna has emerged with the invasion of the Asia-origin snake lung parasite, Raillietiella orientalis (Hett, 1915), into both Australia and the United States (Kelehear et al., 2011; Miller et al., 2018). Raillietiella orientalis causes a disease known as pentastomiasis (specifically, raillietielliasis). This hematophagous, obligate parasite belongs to the crustacean Subclass Pentastomida, which includes a diverse group of respiratory parasites-125 recognized species-distributed across all continents (Poore, 2012; Curran et al., 2014). These parasites typically have heteroxenous life cycles involving both intermediate and definitive hosts (Paré, 2008). In Florida, R. orientalis is commonly referred to as the invasive snake lungworm due to its vermiform shape and its frequent infection of snakes as the definitive host. In Australia, 38% of 81 wild-caught snakes were infected with R. orientalis, including species in the families Elapidae, Colubridae, and Pythonidae (Kelehear et al., 2014). In both locations of R. orientalis invasion, native snakes experience severe infections with up to 77 individuals in the lung in Australia and up to 107 individuals in the lung in the eastern United States (Kelehear et al., 2014; Palmisano et al., 2025). While the invasion pathway into Australia remains unknown, R. orientalis appears to have been introduced to North America via Burmese pythons (Python bivittatus) in south Florida and was first detected in 2012 (Miller et al., 2018).

Raillietiella orientalis is pervasive in the herpetofauna of much of Florida and is associated with pulmonary lesions (*e.g.*, pneumonia), sepsis, and mortality events in several wild and captive snake species (Farrell et al., 2019; Walden et al., 2020; Bogan et al., 2022; Palmisano et al., 2025). Unlike most pentastome parasites which have two-host life cycles, *R. orientalis* utilizes a

three-host life cycle that includes an initial intermediate host (coprophagous invertebrate), secondary intermediate hosts (lizards and frogs), and a definitive reptile host (Palmisano et al., 2022). The definitive hosts are primarily snakes but adult R. orientalis have also been documented in two invasive lizard species (Fieldsend et al., 2021; Goetz et al., 2021). Lung infections by R. orientalis adults are now widespread in native Florida snake species (18 species documented to date; Metcalf et al., 2019; Miller et al., 2020; Bogan et al., 2024; Horvath et al., 2025). The geographic range of these infections is rapidly expanding throughout Florida, with occurrence data from 2024 showing that the range now extends at least 435 km northwest and 80 km northeast of the previous northernmost documentation from 2020 (Palmisano et al., 2023, 2025). Furthermore, R. orientalis is present in the pet trade and has been documented in reptiles that are commonly sold from the wild including banded water snakes (N. fasciata) and tokay geckos (Gekko gecko) (Fieldsend et al., 2021; Farrell et al., 2023). Ongoing geographic and taxonomic spread of R. orientalis infections combined with a poor understanding of host susceptibility within and among species make reliable detection methods essential for understanding the threat of R. orientalis to native herpetofauna and captive animals.

Fecal samples are often used to diagnose parasitic infections, particularly in species that shed eggs in the host's feces (Wellehan and Walden, 2019). A variety of techniques are used including fecal smears, sedimentation, and flotation. The best method of fecal analysis depends on the parasite species of concern (reviewed by Ballweber et al., 2014). Unfortunately, the effectiveness of these diagnostic modalities is not well established for detecting pentastomes in reptiles. Recent studies of R. orientalis infection status in snakes (e.g., Kelehear et al., 2014; Miller et al., 2018; Palmisano et al., 2023) have relied heavily on gross observations and sampling road-killed individuals for parasite identification; however, there is a need for methods that allow for non-invasive, antemortem detection. For most host taxa, freshly produced feces are preferred for diagnostic techniques because sample storage duration often corresponds with reduced parasite detection (Nielsen et al., 2010). Documenting the detection efficacy for both fresh and dried fecal samples is important for pentastome detection in snakes because many species, including several that are common in the pet trade, rarely defecate making fresh sample collection challenging (Lillywhite et al., 2002).

In the eastern United States, snakes can become infected with two native pentastome species, *Kiricephalus coarctatus* and *Porocephalus crotali*, as well as *R. orientalis* (Miller et al., 2018; de Luna et al., 2022). Researchers currently use two conventional PCR assays to identify pentastomes from DNA extracted from adult pentastome tissue. One assay targets the 18S ribosomal RNA gene, which identifies pentastomes to genus level with Sanger sequencing of target amplicons (Brookins et al., 2009). The other assay utilizes the cytochrome c oxidase subunit I (CO1) region and can distinguish among species of pentastomes with Sanger sequencing of target amplicons (Folmer et al., 1994). However, neither assay has been tested for effectiveness of amplifying pentastome DNA from non-invasive sample types such as fecal samples and cloacal swab samples from live hosts. Furthermore, pentastomes are metazoans, which share more genetic similarity with their hosts (*e.g.*, snakes) than do viruses or bacteria. This increased genetic similarity heightens the risk of amplifying host DNA and other invertebrate DNA at conserved gene targets, reducing the utility of these targets for accurate parasite taxonomic identification.

In this study, we describe best practices for non-invasive pentastome egg sampling from snakes and present an optimized PCR protocol specific for the R. orientalis CO1 gene. Specifically, we (1) compare the sensitivity of fresh versus aged fecal samples from snakes with confirmed R. orientalis infections and (2) evaluate different fecal flotation techniques to assess the optimal fecal sample collection and flotation techniques to detect the presence of R. orientalis eggs. We also (3) develop a novel, conventional PCR assay targeting the *R. orientalis* CO1 gene to provide a reliable, costeffective, and easy-to-implement tool for pathogen detection, and (4) assess the effectiveness of various sample types for PCR. The methods presented offer valuable techniques for non-invasive sampling and detection of R. orientalis infections in definitive hosts, providing efficient tools for pathogen surveillance. These advancements will enhance our ability to monitor for R. orientalis in snake populations of the eastern United States, contributing to a better characterization of the threat it poses to native reptiles and potentially informing management efforts.

Methods

Fecal flotation solution comparison

We collected snakes from a site located in Volusia County, Florida which has a high prevalence of R. orientalis (Farrell et al., 2019). We retained snakes in the Reptile Lab at Stetson University for ~1 week to collect fresh fecal samples to detect patent R. orientalis infections via fecal wet mounts. We returned apparently uninfected specimens to their site of capture, and retained four infected snakes, including a black racer (Coluber constrictor, caught 7 September 2023), two banded water snakes (Nerodia fasciata, caught 26 August and 21 May 2023), and a ribbon snake (Thamnophis saurita, caught 10 April 2023). We confirmed these four snakes with pentastome eggs in their feces were infected by R. orientalis by (1) euthanizing, dissecting, and morphologically verifying the pentastome species after the end of the experiment (three snakes), or (2) examining an adult female pentastome expectorated during the experiment (one snake). Adult R. orientalis are readily distinguished from the native pentastome species infecting snakes in North America by head morphology (de Luna et al., 2022).

We housed the snakes individually in glass terraria with heating pads, newspaper substrate, water bowls, and two hiding locations. We fed the snakes once each week using frozen-thawed fish and rodents for *N. fasciata*, rodents and lizards for *C. constrictor*, and lizards for *T. saurita*. We checked terraria daily and collected fresh feces while avoiding urates and portions of feces comprised of mostly fur remnants. We collected 20 fecal samples from 26

September 2023 to 1 February 2024 (eight from *C. constrictor*, one from *T. saurita*, seven from *N. fasciata* #1, and four from *N. fasciata* #2). Immediately after collection, we mixed the feces with a sterilized (cleaned with 70% ethanol solution) metal spatula and subdivided each fecal sample into four similar-sized subsamples that we then weighed on an analytical balance. The fecal subsamples were small (mean mass=68.6 mg) to facilitate complete pentastome egg counts.

For each fecal sample, we randomly assigned one of four diagnostic procedures to the four subsamples. One subsample was used in its entirety to make a series of fecal smear wet mounts to allow us to count the total number of pentastome eggs per gram of fecal material. The wet mounts were prepared by mixing small amount of fecal material with approximately 0.1 mL of tap water on a glass slide. A cover slip was then placed over the mixture. Forceps were used to break apart any large, hard pieces of fecal matter, allowing them to disperse more evenly in the water for improved visualization on the slide. The other three subsamples were used with three flotation solutions, zinc sulfate (ZnSO₄, LabChem, specific gravity=1.18), sodium nitrate (NaNO₃, "FECA-Med", Vedco Inc., specific gravity=1.26), and Sheather's sugar solution (JorVet, Jorgensen Laboratories, specific gravity=1.27). In all flotation tests we placed a subsample in a fecalizer (BioRx Laboratories, Commerce, CA), which is a device consisting of a well and an insert with screening that grinds up the fecal sample and filters the debris from the eggs. One of the three test solutions was added to the fecalizer to the first fill line and the insert was rotated by hand to break up the feces. More solution was added to the fecalizer until the formation of a positive meniscus, at which point a glass cover slip was placed on top. The cover slide remained in position for ten minutes to allow the eggs to rise from the debris and accumulate on the cover slip, which was then carefully removed and placed on a slide. The slide was viewed under 50x brightfield microscopy and the number of pentastome eggs was recorded.

As these snakes were infected with pentastomes, we classified each test as a true positive result if any pentastome eggs were observed in a fecal subsample or as a false negative if no eggs were observed. To calculate the observed pentastome egg density in a fecal sample, we divided total number of eggs counted in the wet mount subsample by the wet mass of that subsample.

Effectiveness of fecal flotation with aged and dried feces

We checked snake cages daily from 30 December 2023 to 7 February 2024 and collected 18 fresh fecal samples (two from *C. constrictor*, six from *T. saurita*, six from *N. fasciata* #1, and four from *N. fasciata* #2). After we retrieved the feces, they were thoroughly homogenized with a spatula. Each fecal sample was separated into two subsamples of approximately equal size and then weighed (mean=89.3 mg, SD=4.9 mg). The subsamples were randomly assigned to either the fresh sample group (analyzed within three days of retrieval) or the aged sample group. The aged samples were kept in a desiccator with relative humidity of zero held at approximately 20°C and were held for an average of 16.5 days (range=14–21 days) before flotation. We used sodium nitrate flotation only (methods as described above) and counted the number of pentastome eggs observed. We divided the egg count by the subsample mass to get an observed egg density for each sample. Samples were classified as either true positive (at least one pentastome egg observed after flotation) or a false negative (no eggs observed).

Raillietiella orientalis and reptile sampling

The R. orientalis samples used for genetic analysis were either opportunistically collected from live reptiles and carcasses and shared via the Snake Lungworm Alliance and Monitoring (SLAM) program (https://invasionscience.ufl.edu/slam/) or collected through other studies. Sample sources included frozenthawed carcasses (i.e., roadkill), live captive-held lizards and frogs (for other studies), and live animals handled for a multitude of other research projects. In total, we tested 611 samples from 27 snake species, four lizard species, and one toad species (Supplementary File 1). These samples were comprised of 412 cloacal swabs and 98 fecal samples from snakes, nine R. orientalis larvae, and 43 R. orientalis adults. Additionally, other pentastome species included 49 adults of two native Florida species, Porocephalus crotali and Kiricephalus coarctatus, collected from snakes, as well as adult Raillietiella females from Hemidactylus spp. These Raillietiella specimens were morphologically consistent with blunt-tipped Raillietiella species (R. orientalis is sharp-tipped), specifically R. indica and R. taegueselfi (de Luna et al., 2022). Their host preference was also consistent with these species and for brevity, we will refer to these specimens as blunt-tipped Raillietiella throughout the manuscript.

Our dataset included 170 *R. orientalis*-negative controls, 155 *R. orientalis*-positive controls, and 286 snakes of unknown infection status. The negative controls included 90 cloacal swabs from carcasses that were confirmed negative, 24 fecal samples with no eggs detected, and samples from adults of three other pentastome species: *K. coarctatus* (n=18), *P. crotali* (n=20), and blunt-tipped *Raillietiella* (n=11), which were identified via morphology (all individuals, n=49) and further validated by DNA sequencing (n=18). Species identification of adults relied primarily on body shape, head, and hook morphology and established life cycles. We used CO1 primers (Folmer et al., 1994) to confirm our morphological identification of four *K. coarctatus*, the three *P. crotali*, and 11 blunt-tipped *Raillietiella* (Supplementary File 2).

Negative controls also included an additional 24 fecal samples that we determined were negative through fecal wet mount microscopy, and 97 cloacal swab samples from snakes and lizards examined post-mortem that had no adult *R. orientalis* in their lungs. Additionally, we extracted DNA from six fecal samples that underwent fecal flotation and kept them separate from our other samples for analysis, due to the potential presence of inhibitors in the flotation solutions (three with sodium nitrate and three with zinc sulfate).

For fecal samples, positive controls included 42 pygmy rattlesnake (Sistrurus miliarius) fecal samples in which we observed pentastome eggs and one carcass that had R. orientalis in the lung. We used Sistrurus miliarius (S. miliarius) fecal samples as positive controls because S. miliarius almost exclusively hosts R. orientalis adult (Miller et al., 2020; Farrell et al., 2019). We confirmed these S. miliarius positive samples via Sanger sequencing (see below). There are currently no known diagnostic features of pentastome eggs that enable species differentiation via our standard wet mount techniques; and therefore, 31 additional fecal samples from other snake host species with pentastome eggs microscopically detected were considered unknowns. For cloacal swabs, the group of positive controls included (1) swabs from snakes that were dissected and found with R. orientalis adults in the lungs, and (2) snakes used in experimental infection studies with confirmed R. orientalis infections via conventional PCR and/or dissections. The positive control pentastome individuals included 9 larval R. orientalis isolated from the viscera of lizards and frogs used in experimental infections, and 43 adult R. orientalis from dissected snakes that were identified morphologically and confirmed via Sanger sequencing.

DNA extractions

For DNA extractions, we used Qiagen DNeasy Tissue kits and adapted the manufacturer's instructions for each sample type (Qiagen, Hilden, Germany). Adult pentastomes were cut with bead-sterilized forceps and scissors, then the 70% ethanol preservative was evaporated off the cut sections. Larval pentastomes preserved in 70% ethanol were placed in 1.5 mL microcentrifuge tubes and ethanol was evaporated off. DNA was extracted from adult (partial) and larval tissue (whole) using the Qiagen DNeasy Tissue kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). For the cloacal swab extractions, we followed the manufacturer's guidelines of placing samples in 20 µL proteinase K and 180 µL of buffer ATL for incubation at 56°C. We incubated the swabs for three to six hours and then proceeded to add 200 µL of 100% ethanol and 200 µL of buffer AL. We used bead-sterilized forceps and scissors to transfer the swabs tipped-end facing up, into the spin columns and then pipetted 600 μ L of the liquid into the spin column. Following the initial spin at 8,000 RPM for one minute, we removed the swabs and followed the manufacturer's guidelines for the remainder of the extractions. The fecal extractions followed the protocol from Ayana et al. (2019), which was developed for the extraction of DNA from helminth eggs in fecal samples. Specifically, we used the modified DNeasy Blood and Tissue Kit protocol without bead beating (Qiagen, Hilden, Germany). We measured the concentrations of all samples using a Nanodrop spectrophotometer, except for three positive fecal samples, one cloacal swab, and one P. crotali sample.

PCR assay development

We initially tested cloacal swabs, fecal samples, and R. orientalis adults with 18S primers (Brookins et al., 2009) and CO1 primers (Folmer et al., 1994) as these were the most recent PCR assays available in the literature. Given the lack of morphological methods for distinguishing pentastome eggs, reliable molecular detection techniques for R. orientalis infections in fecal samples were needed. Because no previous studies had evaluated the effectiveness of cloacal swabs or fecal samples with these assays, we utilized positive control samples confirmed to be infected with R. orientalis via dissection to test these methodologies and determine whether a new assay was needed. Specifically, we ran the 18S assay with 30 adult R. orientalis identified morphologically, 14 fecal samples with pentastomid eggs identified through wet mounts (three were from S. miliarius and considered positive controls, the other 11 were considered unknown), and 16 cloacal swabs, including the four positive control snakes used for the fecal flotation methods. We ran the Folmer CO1 assay with six adult R. orientalis and 16 cloacal swab samples described above. Due to the limited utility of these primer sets (see Results) and because no primers existed that were specific to R. orientalis, we developed an R. orientalis-specific PCR assay.

We focused on CO1 because 18S data in GenBank revealed no SNPs segregating between R. orientalis and other pentastomes. We designed R. orientalis-specific CO1 primers based on available sequences in GenBank. Specifically, we retrieved all available CO1 sequences across pentastomid species in GenBank, including R. orientalis, R. indica (and junior synonym R. hebitihamata), R. mottae, Porocephalus crotali, Armillifer spp., Kiricephalus coarctatus, Linguatula spp., and Waddycephalus spp. We performed multiple sequence alignment on these sequences to identify regions that were identical among all R. orientalis samples but contained single nucleotide polymorphisms (SNPs) that were fixed between R. orientalis and all other pentastome species. We designed primers within regions that had the highest density of these SNPs, corresponding to a target region spanning 194 base pairs of the CO1 gene: forward primer ROCO1F=5'-GCCTTCTCCATATTACTCCTC-3; reverse primer ROCO1R=5'-CGTATGTTGATGATTGTGGTAGTG-3'. The primers were designed for specificity and efficiency, taking into consideration factors such as melting temperature and potential secondary structures.

We optimized the PCR protocol using temperature gradient cycling protocols with *R. orientalis* positive controls and negative controls (including the other pentastome species). For our 20 μ L reactions, we used 1 μ L of template DNA, 0.5 μ l of each primer (ROCO1F, ROCO1R) at 10 μ M concentration, 13.95 μ L of molecular-grade water, 2 μ L of NEB OneTaq Buffer (5x), 0.8 μ L of dNTPs (10mM/dNTP), 0.8 μ L of BSA (10%), 0.2 μ L of DMSO (10mM), and 0.25 μ L of NEB One Taq enzyme. We amplified our 20 μ L mixture in a thermal cycler (Bio-Rad T100) with a 3-minute denaturation period at 94°C, 35 cycles of: denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 60 seconds, and a final extension at 72°C for 7 minutes. To

visualize the amplified product, we performed gel electrophoresis using a 2% agarose gel, run at 80 volts for 45 minutes.

We Sanger sequenced 212 PCR products from our *R. orientalis* protocol that produced bands of the expected size (~200 bp), as well as 22 additional PCR products (7 fecal samples with unknown eggs, 13 unknown cloacal swabs, and three positive control cloacal swabs) that had nontarget bands (larger than expected or smeared). All sequencing was performed by Eurofins Genomics (Louisville, KY, USA).

Statistical analyses

For fecal flotation analysis, we used a 4×2 Fisher's exact test to determine if there was a statistically significant association between diagnostic test outcome (true positive or false negative) and testing method (wet mount, zinc sulfate, sodium nitrate, or Sheather's solution). The egg density data exhibited right-skewed distributions, so we log transformed the data to meet assumptions of parametric statistics before data analysis. We used a general linear model with diagnostic method (wet mount, sodium nitrate float, zinc sulfate float, Sheather's float) as a fixed factor and fecal sample as a random effect to determine if there were significant differences between the mean egg densities observed among the four treatments using JMP v18 (SAS Institute, Cary, NC). A Tukey test was used to determine which methods significantly differed in mean observed egg density. For aged versus fresh fecal analysis, we used a Fisher's exact test to determine if there was a difference in the number of false positive results between fresh and aged samples. We conducted a Passing-Bablok agreement analysis on the egg counts for fresh and dried subsamples followed a Bland Altman analysis in JMP v18. We also determined if the mean egg density differed between the fresh subsamples and the dried, aged subsample groups using a paired t-test.

To analyze the efficacy of our PCR assay, we separated our samples into groups by sample type (i.e., cloacal swabs, eggs in S. miliarius fecal samples, and pentastome adults and larvae) and by R. orientalis infection status (positive or negative). In this approach, each test result was treated independently based on the known infection status of the animal (confirmed by dissection or microscopy of fecal samples from S. miliarius), which allowed us to evaluate assay performance and sensitivity according to sample type. We calculated the sensitivity, specificity, positive predictive value, and negative predictive value for our R. orientalis assay across the three sample types (pentastomes, cloacal swabs, and fecal samples) using the confirmed positive and negative controls. Sensitivity was calculated as the proportion of animals that were correctly identified as infected by our assay (true positives) out of all animals known to be infected (the sum of true positives and false negatives). Specificity was calculated as the proportion of animals that were correctly identified by our assay as uninfected (true negatives) out of all animals that were uninfected (the sum of true negatives and false positives). The positive predictive value was calculated as the proportion of animals that were correctly identified by our assay as positive (true positives) out of all animals that tested positive, regardless of their true infection status. Finally, the negative predictive value was calculated as the proportion of animals that were correctly identified by our assay as uninfected (true negatives) out of all animals that tested negative, regardless of their true infection status. We quantified differences in detection rates between sample types by calculating Relative Risk for independent groups (i.e., cloacal swabs with paired fecal samples vs. non-paired cloacal swabs) and Odds Ratio for paired samples (i.e., fecal vs. cloacal swabs from the same individual). Confidence intervals were derived using the epitools package in R (Aragon, 2020; Posit, 2024; R Core Team, 2024).

For snakes of unknown infection status (*i.e.*, cloacal swabs of live snakes and fecal samples with pentastome eggs that were not *S. miliarius*), we assessed the relationship of DNA concentration and amplification with a density plot. For positive control cloacal swabs, we assessed whether sample DNA concentration was associated with amplification probability using logistic regression. We only performed this analysis on positive control cloacal swabs given that almost all other positive control sample types amplified regardless of DNA concentration. In our analysis, we included a density plot to visualize the distribution of amplified samples relative to their DNA concentrations. The density plot was generated using ggplot2 in R (Wickham, 2016; R Core Team, 2024) and displays the likelihood of amplification across varying concentrations, helping to identify potential thresholds that may enhance amplification success.

Independent PCR assay testing

To further validate our PCR assay, we tested the extraction and amplification protocols in a separate research laboratory at the University of Georgia (Athens, GA) using samples from nine snakes collected from Florida which were determined to have *R. orientalis* infections through postmortem evaluation and morphological pentastome identification. The snake species sampled included the black racer (n=1), common garter snake (n=1, *T. sirtalis*), *S. miliarius* (n=5), banded watersnake (n=1), and corn snake (n=1, *Pantherophis guttatus*). Adult pentastomes were collected and stored in 70% ethanol. Additionally, we collected fecal samples and cloaca swabs from each snake and stored samples at -80°C.

We cut adult pentastomes using sterile razor blades and forceps targeting a midbody section approximately 5 mm in length. Ethanol was evaporated off the cut section and extracted using the Qiagen DNeasy Tissue kit, following the manufacturer's instructions (Qiagen, Hilden, Germany). We prepared fecal smear wet mounts (n=9) using the exact methods as above. We initially extracted fecal samples (n=6, samples with sufficient fecal content remaining following fecal smear wet mounts) using the Zymo Quick-DNATM Fecal/Soil Microbe MiniPrep Kit following manufacturer's instructions (Zymo Research, Irvine, California, USA). We repeated extractions when sufficient fecal material (n=6) remained, using the adapted fecal extraction protocol with the Qiagen DNeasy Tissue kit as detailed above. Cloacal swabs were extracted similarly to the above; however, were vortexed for one minute following the addition of 180 µL of Buffer ATL and 20 µl of

Proteinase K and incubated at 56°C for one hour. Additionally, we performed an extra centrifugation step after the 3-minute centrifugation recommended by the manufacturer and used 100 μ L of elution buffer. We used the same PCR and thermal cycler protocols however, we used two different thermal cyclers (Bio-rad DNAEngine; MJ Research PTC-200), and a different master mix. The master mix consisted of a 25 μ L reaction volume with 5 μ L of template DNA, 0.5 μ L of 50 μ M forward and reverse primers, 11 μ L of molecular-grade water, 5 μ L of Green GoTaq[®] Flexi Buffer (5x), 0.25 μ L of dNTPS (80mM/dNTP), 2.5 μ L of MgCl₂ (25mM), and 0.25 μ L of GoTaq[®] Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA). We Sanger sequenced all PCR products at the appropriate base pair length through Genewiz (Genewiz from Azenta Life Sciences, South Plainfield, New Jersey, USA).

Results

Fecal flotation solution comparison

For fecal sample analysis, all four diagnostic methods resulted in a high frequency of pentastome egg detection. The frequency of false negatives ranged from 0% for the wet mounts to 25% for the Sheather's solution subsamples (Table 1), and these differences were not statistically significant (Fisher's exact test, P=0.071). The mean pentastome egg density observed was significantly affected by diagnostic method ($F_{3,57} = 32.53$, P < 0.0001; Table 1). The mean number of eggs observed was significantly higher in the wet mount subsamples than in the three flotation solutions and the sodium nitrate and zinc sulfate floated subsamples had significantly higher means than Sheather's solution subsamples (Tukey test, Table 1).

Aged versus fresh sample comparison

We observed only a single false negative flotation in the aged fecal subsamples and no false negative results among the fresh subsamples (Table 2) resulting in no significant differences in the frequency of false negative results in the two subsample treatments (Fisher exact test, P=1.00). Agreement analysis for egg counts in the fresh and aged subsamples (Supplementary File 3) indicated a

TABLE 1 Comparison of fecal analyses using wet mounts and three flotation solutions in fecal samples (n=20) for detection of *R. orientalis* egg shedding in snakes.

Diagnostic method	True positives	False negatives	Mean observed eggs/g (SE)
Wet mount	20	0	4893.6 (1894.3)
Sodium nitrate float	19	1	1118.4 (348.3)
Zinc sulfate float	18	2	457.6 (165.7)
Sheather's sugar float	15	5	319.1 (179.6)

TABLE 2 The effect of aging and drying on fecal flotation results and observed *R. orientalis* egg density (all samples done with NaNO₃ flotation).

Fecal type	True positives	False negatives	Mean observed eggs/g (SE)
Fresh	18 (100%)	0 (0%)	1241.0 (520.5)
Dried	17 (94.4%)	1 (5.6%)	366.6 (174.2)

significant bias between the two treatments, with the fresh subsample having higher egg counts than the dried subsample in 14 of 18 comparisons. The mean egg density in the fresh samples (1241.0 eggs/g) was significantly higher than the mean of the aged subsamples (mean = 366.6 eggs/g, t=3.30, df = 17, P=0.004).

General invertebrate PCR assays

We ran the Folmer et al. (1994) CO1 assay with six *R. orientalis* adults, which produced amplicons of 710 bp. DNA sequencing confirmed that all six morphologically identified samples were indeed *R. orientalis* with sequences being 100% identical to other *R. orientalis* CO1 sequences in GenBank (accession number MG559641). Additionally, the three individuals identified as *P. crotali* (n=3) were found to be 99–100% identical to *P. crotali* sequences in GenBank (accession numbers MG559653). We were unable to amplify *R. orientalis* DNA from the 16 cloacal swabs as well as the 11 blunt-tipped *Raillietiella* using this protocol, though we ran the PCR three times with positive controls (pentastome adults of other species) that always produced PCR bands of the expected size (710 bp).

For the 18S assay (Brookins et al., 2009), we successfully produced the target PCR amplicon size of ~425 bp in all cloacal swabs (n=16) and 14 fecal samples. Sequencing of these amplicons from cloacal swabs resulted in host DNA for all cases. For the fecal samples, sequencing results were mixed: three samples revealed host DNA (one of the positive control *S. miliarius* samples), while five samples yielded DNA from helminths (two of the positive control *S. miliarius* samples), despite confirming the presence of pentastomid eggs with wet mounts. Three fecal samples produced no amplicons,

and three others returned sequences that were 100% identical to multiple pentastomid genera.

Notably, all 41 Raillietiellid individuals (*R. orientalis* and blunt tipped *Raillietiella*) amplified with the 18S assay produced amplicons of the target size (~425 bp). However, the returned sequences had 99–100% matches to multiple *Raillietiella* spp. in GenBank. Additionally, 19 of the *R. orientalis* individuals returned sequences identified as *Reighardia* spp. which infect seabirds as definitive hosts. For the other pentastome species, four *K. coarctatus* and three *P. crotali* samples produced amplicons of the target size (~425 bp). Of the *K. coarctatus* samples (n=4), two returned sequences with 99–100% identity to *K. coarctatus* (accession number MG559618), while the other two yielded sequences with 99–100% identity to multiple pentastome genera. Of the *P. crotali* samples, two yielded sequences with 99–100% identity to multiple pentastome genera, and one returned sequence with 99–100% identity only to *P. crotali* (accession number MG559598; Supplementary File 2).

Novel R. orientalis specific PCR assay

Using our novel R. orientalis specific PCR assay, we recovered 100% positive predictive value across all positive controls, including R. orientalis adults, R. orientalis larvae, fecal samples with pentastomid eggs from S. miliarius, and cloacal swabs from snakes confirmed to harbor R. orientalis through dissection or inclusion in experimental infections (Table 3). Additionally, all positive control samples had 100% specificity with zero false positives observed. We did not get amplification of adult samples from pentastome species other than R. orientalis, regardless of their DNA concentration (Figure 1; Supplementary File 4, Figure 1). Negative predictive values were 98% for adult and larval R. orientalis, with all samples amplifying except for one larva. Similarly, the negative predictive value for positive control fecal samples was 96%, with only one fecal sample from a confirmed R. orientalis-positive S. miliarius that did not amplify. With this one false negative, the sensitivity for detection in fecal samples was 97.6%, and with one false negative for individuals with known R. orientalis the sensitivity was 98.1%. In contrast, we recovered 47 false negatives from cloacal swab samples, translating to 67.4% negative predictive value and 21.7% sensitivity (Table 3). We

TABLE 3 The positive predictive values (PPV), negative predictive values (NPV), sensitivity, and specificity for all positive and negative controls used to validate a novel PCR assay for *Raillietiella orientalis* identification.

Sample type	Positive control (proportion amplified)	Negative control (proportion amplified)	PPV (95% CI)	NPV (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Pentastomes (adults & larvae)	52/53	0/49	100% (0.94, 1.0)	98% (0.94, 1.0)	98.1% (0.87, 1.0)	100% (0.93, 1.0)
Feces	41/42	0/24	100% (0.91, 1.0)	96% (0.88, 1.0)	97.6% (0.93, 1.0)	100% (0.88, 1.0)
Cloacal Swabs	13/60	0/97	100% (0.78, 1.0)	67.4% (0.60, 0.75)	21.7% (0.11, 0.32)	100% (0.97, 1.0)

For pentastome samples, positive controls were morphologically and genetically confirmed as *R. orientalis* adults or larva, while negative controls were other species of pentastomes confirmed both morphologically and some genetically. Likewise, fecal, and cloacal positive controls samples were from *R. orientalis*-infected snakes, while negative control samples were from uninfected snakes determined by dissection or absence of eggs in fecal wet mounts.



failed to amplify *R. orientalis* DNA in the six fecal samples that underwent fecal flotation.

Effectiveness of sample type

Raillietiella orientalis individuals (adults and larvae) and fecal samples were more likely to amplify with our assay compared to cloacal swab samples (Table 3). For positive control cloacal swab samples, we did not find a significant effect of DNA concentration on the probability of amplification (Estimate=0.00042, SE=0.0022, z=0.19, P=0.85). The odds ratio for amplification based on DNA concentration was 1.00 (95% CI: 0.99, 1.005) (Figure 2). However, the density plot of amplification from cloacal swabs shows a left skew for samples that did not amplify (Figure 3), suggesting that lower DNA concentrations may reduce the likelihood of amplification (Supplementary File 4, Figure 3). Similarly, fecal samples and cloacal swab samples from hosts with unknown status of *R. orientalis* infection also show that samples that did not amplify were skewed toward lower DNA concentrations (Figure 4; Supplementary File 4, Figure 2).

We collected fecal samples from 139 of the 296 live snakes with cloacal swabs collected. Therefore, these 139 snakes were known to have active fecal content in their cloaca at the time of swabbing. Of these paired samples (fecal and cloacal), 25.2% fecal samples (35/139, 95% CI: 0.18, 0.33) and 7.9% cloacal swabs (11/139, 95% CI:

0.04, 0.14) produced PCR bands of the expected size using our novel assay (Supplementary File 4, Table 1). Fecal samples were ~6 times more likely to test positive than their paired cloacal swabs (OR = 6.27; 95% CI: 2.79–14.06). Among the live snakes with non-paired cloacal swabs, 5.1% (8/157, 95% CI: 0.02, 0.1) yielded PCR bands of the expected size. Among cloacal swabs paired with positive fecal samples, 22.9% were also PCR positive (8/35; 95% CI: 0.10–0.40), compared to only 5.1% in the non-paired group. This represents a 4.94 times higher likelihood of detection via swabs from snakes with paired, positive fecal samples (95% CI: 2.20–11.08).

Sanger sequencing

DNA sequencing with the *R. orientalis*-specific assay confirmed that all samples that produced PCR amplicons of the expected size (Supplementary File 5, Figure 4) produced CO1 sequence data specific to *R. orientalis* (all were 100% identical to other *R. orientalis* CO1 sequences in GenBank). Further, all sequences were 100% identical to each other, suggesting little sequence variation in this gene region, at least within the Florida population of *R. orientalis* (GenBank accession number PQ621873). All samples that amplified nontarget bands returned host (snake) DNA sequences. Further, we duplicated the reaction, gel, and sequencing for 95 positive control samples and received



the sample results for all but one (98.9%). When compared with the results from the University of Georgia laboratory, results from our *R. orientalis*-specific assay were consistent with our initial findings, with 100% of *R. orientalis* adults (n=9), 44.4% of cloacal

swabs (n=9), and 85.7% of fecal samples (n=7) producing the target amplicon, and all other samples producing no amplicons. Sequences from these samples were 100% identical to *R. orientalis* sequences in GenBank (Table 4).



The density of *Raillietiella orientalis* DNA concentrations among positive controls of three sample types with amplification status. One larval *R. orientalis* and one fecal sample did not amplify and are not included due to low sample size (n=1). The larva had a concentration of 3.3 ng/µL and the fecal sample had a concentration of 4.9 ng/µL.



Discussion

Our study describes optimized pentastome sampling methodology for snake feces and a novel *R. orientalis*-specific PCR assay that produces ~200 bp PCR amplicons only if *R. orientalis* DNA is present (Supplementary File 4, Figure 4). Our PCR protocol is a robust and cost-effective detection tool that has 100% specificity across all sample types. We validated this protocol for use in detecting *R. orientalis* by testing 611 samples from 27 snake species, four lizard species, and one anuran species, including 155 confirmed positive control samples and 170 confirmed negative control samples. Consistent results obtained from a second laboratory, where different reagents, equipment, and researchers were involved, further validate the protocol's reproducibility, and highlight its potential for widespread adoption across diverse research settings.

This assay is inexpensive and straightforward to use for anyone with access to DNA extraction and PCR equipment. Quantitative (q)PCR assays are incredibly useful for microparasitic pathogens as they are sensitive and provide information on pathogen burdens that cannot otherwise be observed. However, qPCR reagents are significantly more expensive than conventional PCR, qPCR requires more expensive equipment, and qPCR is less relevant for macroparasitic pathogens where quantification is not plausible from indirect samples (and can be measured visually from direct samples). Thus, we focused on a simple diagnostic that reliably confirms the presence or absence of *R. orientalis*.

If the goal is to identify *R. orientalis* presence in fecal samples, adult or larval pentastomes, or cloacal swabs in the eastern United

States, we recommend using our protocol. If amplification does not occur via our protocol with adult or larval pentastomes, we recommend using the Folmer CO1 assay for parasite species identification outside of *R. orientalis*. However, nontarget amplification is possible based on our CO1 endosymbiont sequences produced from unknown Raillietiellid samples. More data are needed on use of the Folmer CO1 assay with cloacal swabs and fecal samples to know if it is reliable in amplifying only pentastome DNA. We recommend using the 18S protocol for exploratory measures of unknown pentastomes but acknowledge its high rate of amplifying non-target DNA due to the lack of genetic differentiation among species at this target region. While our assay is reliable for diagnostic purposes, it is not useful for analysis of *R. orientalis* genetic variation as it contains no polymorphisms. For examining different genetic lineages, the Folmer CO1 primers are a better tool.

Our study had several limitations. First, we had a low rate of amplification from positive control cloacal swabs. However, considering the relative ease and non-invasive nature of cloacal swab collection, and the 100% specificity from cloacal swabs, we recommend further investigation into the potential use of pooled cloacal swab samples as a tool for *R. orientalis* surveillance. While we did not assess the impact of pooling on sensitivity or specificity, we believe this approach may be worth considering in future studies, especially for large-scale surveillance efforts. However, because cloacal swabs have a high false negative rate, many swabs should be analyzed before determining a location is free of the parasite. We recommend the use of fecal samples when available, as TABLE 4 PCR and fecal smear wet mount results from the nine independent snakes used for external validation at the University of Georgia.

Sample type	Proportion detected		
Adult pentastomes	100% (n=9/9)		
Feces using Zymo ^a	83.3% (n=5/6)		
Feces using Qiagen ^b	83.3% (n=5/6)		
Feces Overall	85.7% (n=6/7)		
Cloacal Swabs	44.4% (n=4/9)		
Fecal Smear Wet Mounts	100% (n=9/9)		

^aZymo Quick-DNATM Fecal/Soil Microbe MiniPrep Kit instructions (Zymo Research, Irvine, California, USA).

^bQiagen DNeasy Tissue kit following manufacturer's instructions (Qiagen, Hilden, Germany). Sample types included adult pentastomes, feces, and cloacal swabs. Feces were extracted using two different extraction kits and were split by brand. Prevalence of successful amplifications and fecal smear wet mounts are calculated as percentages and given as fractions of snakes with detection over total number of snakes sampled. PCRs for the different sample types were all processed using the novel CO1 *Raillietiella orientalis*-specific primers developed in this study.

our data showed that fecal samples from infected snakes were more likely to detect *R. orientalis* than their paired cloacal swabs. The higher prevalence of *R. orientalis* detected from cloacal swabs in snakes with paired fecal samples indicates that the presence of fecal content in the cloaca may increase sensitivity. Further research should investigate the effectiveness of cloacal swabs with varied DNA extraction methods and their temporal relation to the last defecation, as fecal samples are often not available from wild, fieldsampled snakes.

Another limitation of our study is that one of nine *R. orientalis* larvae and one of 44 fecal samples with *R. orientalis* eggs did not amplify using our assay. Since we tested a small sample of larvae (all from the secondary intermediate host), our results raise questions about the reliability of larval amplification. Future research should investigate how sensitive the assay is with larvae and adults that have lower than five μ L of DNA, as we only had six out of 52 samples that had lower than five μ L, and across fecal samples with various egg densities upon wet mount. Additionally, further studies are needed to determine whether fecal flotation techniques inhibit PCR amplification, or if our small sample size led to a possible type II statistical error. For now, we recommend saving a portion of the fecal samples before flotation for DNA extraction and prioritizing fecal samples over cloacal swabs.

A third limitation to our study and for all pentastome parasite research is the lack of genetic and taxonomic information for this group. Of the 42 recognized *Raillietiella* species (Poore, 2012), most lack comprehensive morphological descriptions and only four species have publicly available molecular data: *R. orientalis* (with two potential but not confirmed junior synonyms, *R. piscator* and *R. gowrii*), *R. indica* (junior synonyms, *R. hebitihamata* and *R. frenata*), *R. hemidactyli*, and *R. mottae*. While we assessed the use of our assay with the congener blunt-tipped *Raillietiella*, further studies are needed using type specimens from additional *Raillietiella* species. This will be essential to confirm the specificity of the assay for *R. orientalis* and to ensure its broad applicability outside of the eastern United States. We need further molecular characterization of pentastomes and the continued development of specific assays to be used for reliable speciation.

Our fecal smear analyses yielded no false negative results, indicating that the infected snakes used in our egg detection studies consistently had eggs detected in their fecal samples. All three of the flotation solutions we used also typically detected eggs in known infected snakes (i.e., true positive test results). We had false negative results in only 9.4% (9/96) of the flotation tests run in the fecal float comparison experiment and the aged versus fresh sample experiment with five of these false negative tests occurring when we used Sheather's sugar solution. Our estimates of the percent of eggs in a sample that were observed after flotation indicated that Sheather's sugar was significantly less effective in harvesting eggs than sodium nitrate and zinc sulfate. Flotation in sodium nitrate (specific gravity 1.25-1.27) was effective in detecting pentastome infection given both the low number of false negative results and the high effectiveness in harvesting eggs. For sodium nitrate flotation, the false negative rate was only 3.6% (2 of 56 tests) for the two experiments. Sheather's solution might be more effective if combined with centrifugation, but this technique requires more equipment and is time intensive compared to the rapid and simple flotation methods we used. We also used small amounts of fecal material, (~<0.1 g), in our flotation methods as we subdivided the feces and intentionally used small samples to facilitate complete egg counts on fecal smear slides. The probability of false negative results may have been lower had we used greater mass of fecal samples.

An additional limitation to this study was the lack of fecal examination through sedimentation techniques. While fecal sedimentation is the preferred technique for detecting trematode larvae and ova (Hayider et al., 2018) and acanthocephalan ova (Nabi et al., 2015), it has not been shown to be better than flotation for nematode ova or protozoan oocysts (Ines et al., 2016). In reptiles, a comparison of fecal examination techniques has been performed for multiple parasites, including pentastomes (Wolf et al., 2014). While Wolf et al. (2014) detected pentastomid ova in both the flotation and the sedimentation techniques, the number of animals with pentastomiasis was too small to allow for adequate statistical comparison. However, we feel that the likelihood of Raillietiella ova detection through a sedimentation technique may be lower than that of the flotation technique since the ova of R. orientalis demonstrated positive buoyancy with a high rate of detection. Further investigation is warranted to evaluate the utility of fecal sedimentation as it relates to fecal flotation for the detection of R. orientalis.

Aging and drying fecal samples for several weeks significantly reduced pentastome egg counts but did not significantly increase the frequency of false negative results. However, this method provides an alternative for use in captive snakes, in which collection of wet fecal samples can be challenging. This is because the period between successive defecation events typically exceeds one month, especially in large constrictors commonly used in the pet trade (Lillywhite et al., 2002). Furthermore, our results indicate that fecal samples from snakes used in field studies can be stored at ambient temperature until processing later. A limitation of the fecal analysis we used is that it will only work for patent (egg bearing) infections and, therefore, cannot detect juvenile-only or male-only infections.

The R. orientalis-specific assay and optimized fecal sample processing will facilitate new research on detecting R. orientalis in wild herpetofauna across the eastern United States. The PCR assay has already expanded our understanding of R. orientalis epizoology, identifying infections in 18 additional Florida counties (Palmisano et al., 2022, 2025) and several new host taxa (Horvath et al., 2025; Palmisano et al., 2025). We recommend fecal samples as the primary detection method in live snakes, and if fecal flotation is used, splitting samples for both flotation and molecular analysis. While wet mounts are effective, they can be difficult with clumped feces containing mammalian hair; in such cases, flotation may be a simpler alternative. If the sample consists mainly of urates, the absence of feces makes it difficult to draw conclusions about infection, as eggs are typically found in fecal material. Fecal samples can be preserved either by freezing (following our University of Georgia protocol) or by storing in 70-90% ethanol at room temperature, protected from direct sunlight. If fecal samples are unavailable, cloacal swabs can be used, but negative results should not be interpreted as indicating the absence of infection. Our study presents robust fecal sampling and PCR protocols for detecting and monitoring R. orientalis infections, contributing valuable data for pathogen surveillance in the eastern United States. These tools can enhance reptile disease research and inform proactive biosecurity measures.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by University of Central Florida, Stetson, and University of Georgia Institutional Animal Care and Use Committees. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JP: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. CH: Data curation, Investigation, Methodology, Validation, Visualization, Writing – review & editing. JG: Data curation, Writing – review & editing. JH: Data curation, Writing – review & editing. TF: Data curation, Methodology, Resources, Writing – review & editing, Formal analysis, Investigation, Writing – original draft. JB: Resources, Validation, Writing – review & editing, Data curation, Methodology. NN: Resources, Supervision, Validation, Writing – review & editing. AS: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. AS: Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/famrs.2025. 1531792/full#supplementary-material

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14