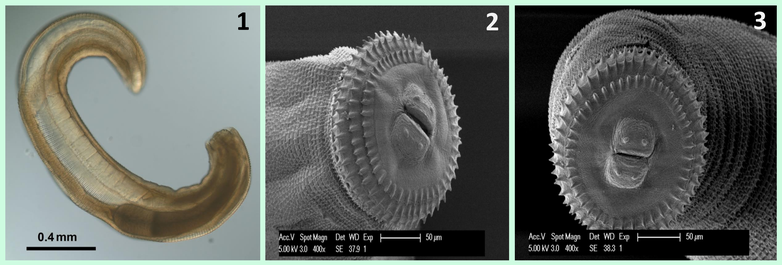
Genetic Characterization and DNA Barcoding of the Zoonotic Human Parasite *Gnathostoma*

Ryan Pogodzinski and Anindo Choudhury
Division of Natural Sciences, Saint Norbert College, De Pere, WI

INTRODUCTION

Zoonotic diseases are those that are caused in humans by parasites and pathogens normally found in wild and domesticated animals. One such disease, gnathostomiasis, is caused by larvae of parasitic nematodes (roundworms) in the genus *Gnathostoma* (Figs. 1-3). These roundworms are found as adults in several carnivorous and omnivorous mammals. Intermediate hosts such as fish and amphibians transmit the infective third larval stages (L3) to the final host through the food chain. Humans become infected by consuming undercooked freshwater fish containing the L3 larvae (Fig. 4). Finding themselves in an abnormal host, these larvae migrate in the body and cause pathology. In recent years, gnathostomiasis has become a serious emerging disease (Fig 5).



Figures 1 - 3: Infective larval stage of *Gnathostoma* from non-native swamp eels: **1:** Whole larva (light microscopy). **2 &3:** Scanning Electron Microscopy (SEM) images of anterior ends of larvae showing rows of spines on the cephalic bulb (Cole, Choudhury, Nico and Griffin, 2014).

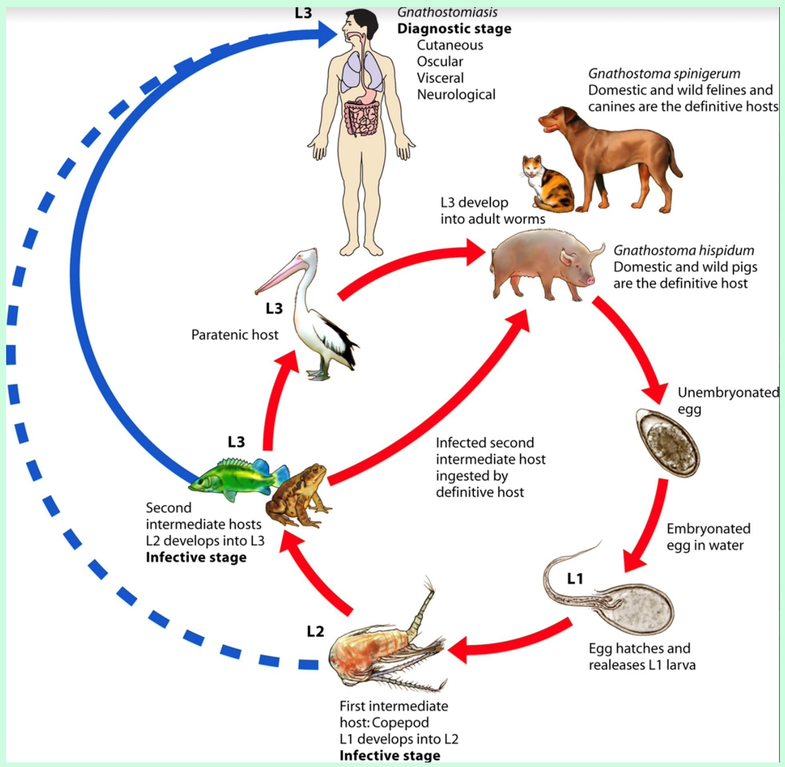


Figure 4. Life Cycle of *Gnathostoma* spp. and transmission to humans (CDC).

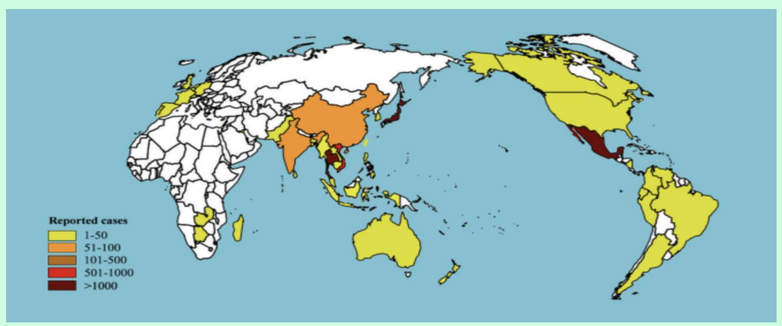


Figure 5. Prevalence of human gnathostomiasis worldwide (Liu et al., 2020)

RATIONALE & OBJECTIVES

Cole, Choudhury, Nico and Griffin (2014) isolated and identified at least three species of *Gnathostoma* from imported and invasive swamp eels, using morphology (Fig. 6) and DNA sequence data from the ITS region of the rRNA gene array.
This study extends the 2014 study with the following **objectives** :

1. DNA barcoding (Hebert & Gregory, 2005) of the three target species of *Gnathostoma* found in the 2014 study, using nematode-specific primers.
2. Generate sequences of the 28S rRNA gene to provide a more complete genetic characterization of these zoonotic nematodes.

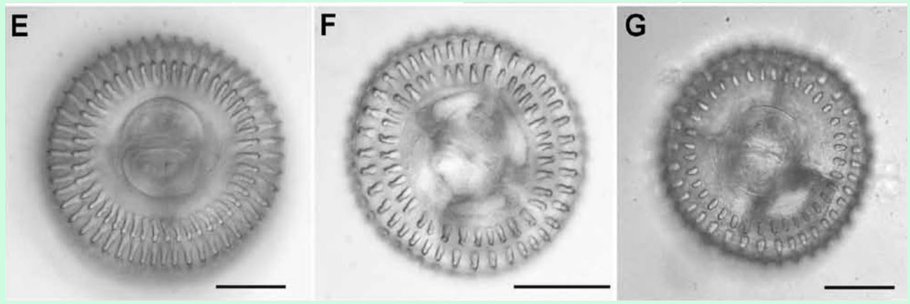


Figure 6. *En face* mounts of the cephalic bulbs from the 3 species represented in this study **E**: *Gnathostoma spinigerum,* **F:** *Gnathostoma turgidum,* and **G**: *Gnathostoma lamothei.* (reproduced from Cole et al. 2014).

MATERIALS & METHODS

- Extracted DNA samples from the 2014 study were provided by Dr. Rebecca Cole (USGS National Wildlife Health Center, Madison, WI).
  - From 26 DNA samples provided, 8 extracts from 3 different species were used (Fig. 6, Table 1).
- DNA concentrations were measured using a Nanodrop Spectrophotometer.
- Polymerase Chain Reaction (PCR) was used to amplify the (1) 28S, (2) CO1, and (3) CO2 genes using the following primer sets: (1) 391a/501, (2) 507F/HCO, and (3) 211F/210R. (Folmer et al., 1994; Nadler et al., 2000; Carreno & Nadler, 2003; Nadler et al., 2006).
  - Thermocycling parameters varied for each separate PCR.
- Amplified products were run on a 1.5% TAE agarose gel stained with GelRed® Prestain Loading Buffer with Tracking Dye (Biotium, Fremont, CA). (Fig. 7)
- Samples were purified using ExoSAP-IT PCR Product Cleanup and sent for sequencing to MCLab (South San Francisco, CA).
- Sequences were assembled and consensus sequences were aligned and analyzed using Mega 11 (Tamura et al., 2021).

Specimen	Extract ID #	Nanodrop on Extracted DNA (ng/uL)	28s Amplification (391a/501 primers)	CO1 Amplification (507F/HCO primers)	CO2 Amplification (211F/210R primers)	CO2 Amplification (211F/210R primers)
Gnathostoma spinigerum	Px622	2.9	+	+	-	+
Gnathostoma spinigerum	Px630	3.5	+	+	-	+
Gnathostoma spinigerum	Px632	6.4	+	+	-	-
Gnathostoma spinigerum	Px633	2.7	+	+	-	+
Gnathostoma lamothei	Px642	3.7	+	+	-	+
Gnathostoma turgidum	Px637	2.8	+	+	-	+
Gnathostoma turgidum	Px638	3.1	+	+	-	-
Gnathostoma turgidum	Px641	3.2	+	+	-	+

Table 1. Table of corresponding DNA extract specimens with Nanodrop DNA concentrations and PCR amplification results.

RESULTS

PCR Amplification:
In total, 32 total amplifications were performed; 8 for 28S, 8 for CO1, and 16 for CO2 (separated into two separate trials of 8 amplifications each).

28S rRNA Gene:

- 100% of the amplifications were successful, but 25% showed light mispriming and 37.5% showed heavy mispriming (Table 2).
- Of the available 28S sequences, 37.5% could be assembled for consensus sequences.

CO1 Gene:

- 100% of the amplifications were successful, but 12.5% showed heavy mispriming.
- CO1 sequences proved to be the most successful, at a 75% success in creating consensus sequences.

CO2 Gene:

- The first 8 amplifications of the CO2 gene showed a 0% success rate, so PCR protocols were modified.
- CO2 amplification proved to be the least successful, with 75% amplification success in the second round, with 66.7% showing heavy mispriming.
- CO2 sequences were not generated.

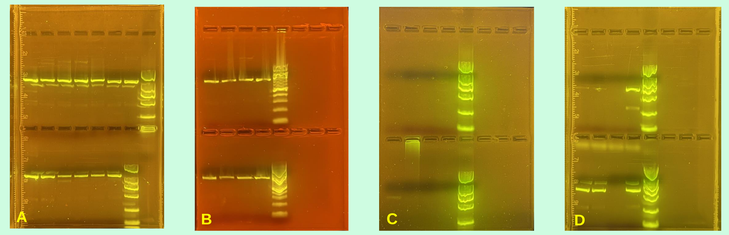


Figure 7. Gel electrophoresis images for selected gene amplifications using PCR. (A) 28S Amplification. (B) CO1 Amplification. (C) First CO2 Amplification. (D) Second CO2 Amplification.

Sequence Analysis:
DNA sequences of the CO1 gene (Fig. 8) and 28S rRNA gene from samples of the three separate species were aligned and analyzed.
CO1 gene (434 sites): Pairwise distance analysis (MEGA 11) showed 0-5 bp difference within species but 49-63 bp differences between two species.
28S rRNA gene (804 sites)**:** Pairwise distance analysis (MEGA XI) showed 0-1bp difference within species but 23-30 bp differences between two species.

Figure 8. Aligned DNA Sequences of the CO1 gene showing differences among the three species.

CONCLUSIONS

- Both CO1 (barcoding) and 28S rRNA genes could be used to distinguish among the 3 species.
- The CO1 gene was readily amplified and showed greater differences among species than the 28S rRNA gene.
- The CO1 gene was validated as a barcoding gene for these species.

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References: Carreno and Nadler 2003; *J. Parasitol*. 89(5):965-73; **Cole, Choudhury, Nico, and Griffin** 2014, *Emerging Infectious Diseases*, 20 (4):634-642; **Liu et al.** 2020; *Parasites and Vectors*. 13:1-11; **Folmer** et al.1994, *Mol. Mar. Biol. Tech*. 3:294-299; **Hebert and Gregory** 2005; *Syst. Biol*. 54(5):852-859; **Nadler & Hudspeth** (2000) *J. Parasitol*., 86(2): 380–393; **Nadler, Bolotin & Stock**. 2006. *Sys. Parasitol*. 63:161–181; Tamura, Stecher & Kumar (2021), *Mol. Biol. Evol*. 38: 3022–3027,